GENETIC ANALYSIS OF NBS-LRR GENES AND THEIR ASSOCIATION WITH ASCOCHYTA BLIGHT RESISTANCE IN CHICKPEA (*CICER ARIETINUM* L.)

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By

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ABSTRACT

Ascochyta blight is one of the major diseases of chickpea worldwide. Genetic control of resistance to ascochyta blight in chickpea is complex and governed by multiple quantitative trait loci (QTLs). The molecular mechanism of quantitative resistance to ascochyta blight and the genes underlying the QTLs are still unknown. The most predominant resistance (R)-genes in plants contain nucleotide binding site and leucine rich repeat (NBS-LRR) domains. One hundred twenty-one NBS-LRR genes were identified in the chickpea genome. Ninety-eight of these genes contained all essential conserved domains while 23 genes were truncated. These NBS-LRR genes were grouped into eight distinct classes based on their domain architecture. Phylogenetic analysis separated these genes into two major groups based on their structural variations, the first group with a toll or interleukin-1 like receptor (TIR) domain and the second group either with or without a coiled-coil (CC) domain. The NBS-LRR genes are unevenly distributed across the eight chickpea chromosomes and nearly 50% of the genes are present in clusters. The expression profiles of thirty NBS-LRR genes that were co-localized with nine previously reported ascochyta blight QTLs were evaluated for potential involvement in response to ascochyta blight infection. Expression patterns of these genes were studied in two moderately resistant ('CDC Corinne' and 'CDC Luna') and one susceptible ('ICCV 96029') genotypes at different time points after ascochyta blight infection using real-time quantitative PCR. Twenty-seven NBS-LRR genes showed differential expression in response to ascochyta blight infection in at least one genotype at one-time point. The majority of these NBS-LRR genes showed differential expressions after inoculation in the resistant and susceptible genotypes suggesting the potential involvement of these genes in response to *Ascochyta rabiei* infection. Five NBS-LRR genes showed genotype-specific expression.

Eighteen QTLs for ascochyta blight resistance were identified in three recombinant inbred line populations (CPR-02, CPR-03, and CPR-04) under greenhouse and field conditions. These QTLs were distributed across linkage groups 2, 3, 4, 5, 6 and 8 explaining 6 to 33% of the phenotypic variations. The current study identified new and common genomic regions associated with ascochyta blight resistance. Thirty-one NBS-LRR genes were co-localized with the ascochyta blight resistance QTLs in four RIL populations. Eight NBS-LRR genes were common in at least two RIL populations. The co-localization of the NBS-LRR genes within the ascochyta blight resistance QTLs was further confirmed by genetic mapping of the NBS-LRR genes in two RIL populations.

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Above all, Thank you to my family, especially Mom and Dad, and my brother Raman for your love, support, and encouragement at all times.

DEDICATION

I would like to dedicate this thesis to all farmers from across the world for their immense contribution in feeding the world.

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CHAPTER 1

INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Chickpea (*Cicer arietinum* L.) is an economically important pulse crop grown in over 50 countries globally. Canada is among the top ten chickpea producing countries in the world (FAOSTAT, 2014). Saskatchewan produces more than 90% of Canada's chickpea and the total area under chickpea production in Saskatchewan ranged from 64,749 ha in 2016 to over 153,780 ha in 2007 (Specialty Crop Report, 2016). Ascochyta blight caused by the fungus *Ascochyta rabiei* (Pass) Labrousse and the short growing season are two major challenges of chickpea production on the Canadian Prairies, limiting the crop's potential to achieve its maximum yield (Gan et al., 2006).

Ascochyta rabiei infects all above ground parts of the chickpea plant which can result in total crop loss if favorable environmental conditions prevail for its infection and further growth (Reddy and Singh, 1990). The disease can be controlled by intensive fungicide applications, but these are not always cost-effective. In addition, high use of certain fungicides such as the strobilurin class can result in insensitivity of the fungus making the fungicides ineffective (Gan et al., 2006). Other agronomic and cultural practices such as crop rotation and seed treatments are helpful in decreasing disease pressure; however, the use of resistant varieties is considered the most efficient and effective way to manage ascochyta blight in chickpea (Gan et al., 2006).

The success in breeding for ascochyta blight resistance has been slow due to the complex nature of the resistance, the limited knowledge of pathogen biology and host-pathogen interaction (Rubiales and Fondevilla, 2012), and the lack of complete resistance in germplasm collections (Singh and Reddy, 1993). Better knowledge of the genetics of the resistance could help to accelerate the breeding process to develop ascochyta blight resistant varieties (Rubiales and Fondevilla, 2012). Thus, there is a need to study the genetics of resistance and to identify potential candidate genes associated with ascochyta blight resistance in chickpea.

Several quantitative trait loci (QTLs) for ascochyta blight resistance have been identified across all chickpea chromosomes in different genetic backgrounds (Anbessa et al., 2009; Cho et al., 2004; Iruela et al., 2006; Santra et al., 2000; Udupa and Baum, 2003). Most of these QTLs were identified using simple sequence repeat (SSR) markers. The success in introgression and selection for these QTLs has been limited due to the large size of the QTL

interval and disassociation of the linked markers from the resistance locus through recombination (Li et al., 2017; Rubiales and Fondevilla, 2012). The availability of the chickpea whole genome sequence facilitated the identification of candidate resistance genes within the QTL regions (Varshney et al., 2014a). By integrating the physical map with two genetic maps using 245 bacterial end-sequence derived simple sequence repeat markers, 306 candidate genes present in the vicinity of ascochyta blight resistance QTLs were identified (Varshney et al., 2014a). Two candidate genes for ascochyta blight resistance, *CaETR1* (*EIN-4 like*) and *ethylene insensitive 3-like gene* (*Ein3*), were identified within the QTL_{AR1} on linkage group (LG) 4 and the QTL_{AR3} on LG 2, respectively (Madrid et al., 2012, 2014). Recently, genome-wide association analysis using whole genome sequence data of 69 chickpea genotypes combined with expression analysis identified a Serine/threonine Receptor-Like Kinase class (RLK) gene as a potential candidate gene for ascochyta blight resistance (Li et al., 2017). These recent studies demonstrate the shift in research from QTL identification to candidate gene discovery based on the availability of the genome sequences. Nevertheless, the underlying mechanism of resistance to ascochyta blight is still unclear.

Plant disease resistance genes or R-genes are a gene family known to code for resistance to diverse plant pathogens (Jones and Dangl, 2006). Among the five classes of R-genes, the largest class encodes a protein containing Nucleotide Binding Site (NBS) and Leucine Rich Repeat (LRR) domains, thus, they are called NBS-LRR genes (Young, 2000). NBS-LRR genes are known for the recognition of effector molecules from the pathogen and further activate the defence reaction to limit pathogen growth (Jones and Dangl, 2006).

The involvement of NBS-LRR genes in resistance to a wide range of pathogens makes these genes an important target for the improvement of disease resistance in plants (Marone et al., 2013). Generally, NBS-LRR genes provide resistance against biotrophic pathogens (Glazebrook, 2005); however, some recent studies have also shown the involvement of NBS-LRR genes in resistance reaction against necrotrophic pathogens (Staal et al., 2008; Zhu et al., 2016). As one of the largest gene families in plants and with a well-characterized function in disease resistance, the NBS-LRR gene family has often been the target for candidate gene identification (Meyers et al., 2003). Therefore, it is important to characterize this gene family in chickpea and examine their potential involvement in response to ascochyta blight infection.

1.2 HYPOTHESIS

NBS-LRR genes contribute in providing resistance against *A. rabiei* infection in chickpea.

1.3 OBJECTIVES

1) To examine the structural diversity, conserved domain architecture and location of NBS-LRR genes in the chickpea genome.
2) To determine the co-localization of the NBS-LRR genes with the ascochyta blight resistance QTLs.
3) To examine the involvement of NBS-LRR genes in response to ascochyta blight infection in chickpea.
4) To identify new and common genomic regions involved in ascochyta blight resistance across three RIL populations in chickpea.
5) To validate the co-localization between NBS-LRR genes with ascochyta blight resistance QTLs intervals.

The research is presented in two chapters. In Chapter 3, NBS-LRR gene homologs were identified and classified in the chickpea genome followed by analysis of structural diversity, motif and domain architecture, as well as genomic distribution. The previously reported QTLs for ascochyta blight resistance were anchored on the physical map of the 'CDC Frontier' genome to identify the co-localized NBS-LRR genes. The selected NBS-LRR gene expression profiles were examined using chickpea genotypes with established differences in the level of resistance against *A. rabiei.*

In Chapter 4, QTLs for ascochyta blight resistance were identified in three chickpea recombinant inbred line (RIL) populations. The QTLs were compared with the previously reported QTLs based on their physical positions to identify new and common genomic regions associated with ascochyta blight resistance. The co-localized NBS-LRR genes were identified within the QTL regions in these RIL populations. Genetic mapping of the selected NBS-LRR genes in two RIL populations was conducted to confirm their physical co-localization within the QTL intervals.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2: LITERATURE REVIEW

2.1 CHICKPEA

Chickpea (*Cicer arietinum* L.) is a self-pollinated, diploid (2n = 2x = 16) species with a genome size of 738 Mb (Arumuganathan and Earle, 1991). The genus *Cicer* belongs to the family Leguminoseae, sub-family Papilionaceae, and a tribe of its own, Cicereae. The genus *Cicer* comprises 43 species; 34 wild perennials and 9 annuals (Van der Maesen, 1987). *Cicer arietinum* L. is the only widely cultivated species within this genus. *Cicer reticulatum* L. which is interfertile with cultivated *Cicer arietinum* L. has been placed in the primary gene pool, whereas *C. echinospermum* D. is placed in the secondary gene pool as it is crossable with both wild *Cicer reticulatum* L. and cultivated *Cicer arietinum* L. All other species are in the tertiary gene pool that are not crossable with cultivated species (Ahmad et al., 2005; Gupta et al., 2016).

Chickpea is one of the earliest domesticated grain crops of the old world that has been cultivated since 7,000 B.C (Zohary and Hopf, 2000). The present day cultivated chickpea is believed to have been domesticated from *Cicer reticulatum*, a wild progenitor from the fertile crescent area of Southeast Turkey and an adjoining part of Syria, which is considered as the center of origin for this crop (Ladizinsky and Adler, 1976). After its domestication in the Middle East, its cultivation spread to the Mediterranean region, Central and South Asia and East Africa. Later, this crop was introduced into South America, North America and Australia.

Cultivated chickpea can be classified into two types: desi and kabuli based on the variability in seed characteristics and flower color. Based on linguistic evidence, the larger seeded chickpea reached India via Kabul (Afghanistan's capital) more than two centuries ago and acquired its name as kabuli chana (chickpea) whereas the small seeded chickpeas with dark seed coat were called desi, which means ‘local’ (Singh, 1997). These two types of chickpea represent different genetic backgrounds differing in important agronomic traits. The desi type chickpea varieties have purple flowers, small angular seeds with a thick dark seed coat, anthocyanin pigmentation on stems and are mainly grown in the semi-arid and tropical regions. In contrast, kabuli chickpea has white flowers, large bold seeds with thin white seed coats (low-tannin), lack of anthocyanin pigmentation on stems and are usually grown in temperate regions. Natural mutation and selection for traits such as seed and flower color from desi type resulted in kabuli type (Singh, 1997). The desi type accounts for about 80-

85% of the total chickpea area and is mainly grown in Asia and Africa. The kabuli type is largely grown in North Africa, North America and Australia (Pande et al., 2005). Local food preferences play a key role in the spread and adaptation of these types in different regions (Singh, 1997).

Chickpea is mainly cultivated for its edible seed, which is a rich source of high-quality protein (25-28.3%), carbohydrates (60.7%), total dietary fiber (17.4%), oil (3.8-10%) as well as various amino acids and polyphenol compounds (Jukanti et al., 2012). Chickpea seeds are a relatively inexpensive source of protein and they are an important component of diets of individuals who cannot afford animal proteins or those who are vegetarian by choice. Therefore, chickpea is particularly important for populations in several developing countries where it is mainly consumed as dal along with rice. Due to its nutritional properties there has been an increase in demand of chickpea in North America and Europe recently, where it is used as the main ingredient of hummus (Wallace et al., 2016). Chickpea is rich in minerals such as calcium, magnesium, zinc, iron, phosphorus and folate, and is low in polyunsaturated fatty acids (Jukanti et al., 2012). Chickpea consumption, even as a small proportion of the diet, is known to improve insulin secretion and decrease the blood cholesterol level (Pittaway et al., 2007). Although chickpea seeds are extensively grown for human consumption, chickpea crop residue is a good source of animal feed (Pande et al., 2005).

Chickpea is mainly grown in semi-arid and temperate regions of the world with more than 81% of the production in India, Pakistan and Myanmar. India is the major chickpea producer contributing 66% of global production (FAOSTAT, 2014). Canada is among the top ten chickpea producing countries which also include Australia, Myanmar, Ethiopia, Turkey, Pakistan, Iran, Mexico, and the USA. Chickpea is relatively tolerant to drought and other abiotic stresses, so it can be cultivated successfully on residual soil moisture in arid and semi-arid regions of the world (Varshney et al., 2014a). This crop is now cultivated in over 57 countries around the globe and during the past 30 years (1984-2014) chickpea production has increased from 6.5 million tons to 13.7 million tons per year (FAOSTAT, 2014). The increases in chickpea production could be attributed to its cultivation in countries such as Australia, Mexico, Canada, Southern Europe and the North-Western USA, in recent decades. With the increase in area and production, chickpea is now the second largest legume crop after soybean in terms of area, and it ranks third after soybean and dry bean in terms of production. In 2016, the total chickpea area harvested was 12.7 Mha and annual production reached 12 million tons worldwide (FAOSTAT, 2017).

Chickpea is a relatively new pulse crop in Western Canada since its cultivation only began in the 1990's with a relatively small area (800 ha in 1995). Its area under cultivation grew rapidly over the following years (**Figure 2.1)**.The area under chickpea cultivation peaked in 2001 and in that year Canada was the third largest producer of chickpea after India and Turkey with a production area of 467,400 ha (FAOSTAT, 2001). It is primarily (88%) grown in the Brown and Dark Brown soil zones of south-western Saskatchewan and south-eastern Alberta in rotation with cereal and oilseed crops (**Figure 2.2)**. The kabuli type is best adapted to the Brown soil zone and desi type to Brown and Dark Brown soil zones of Saskatchewan.

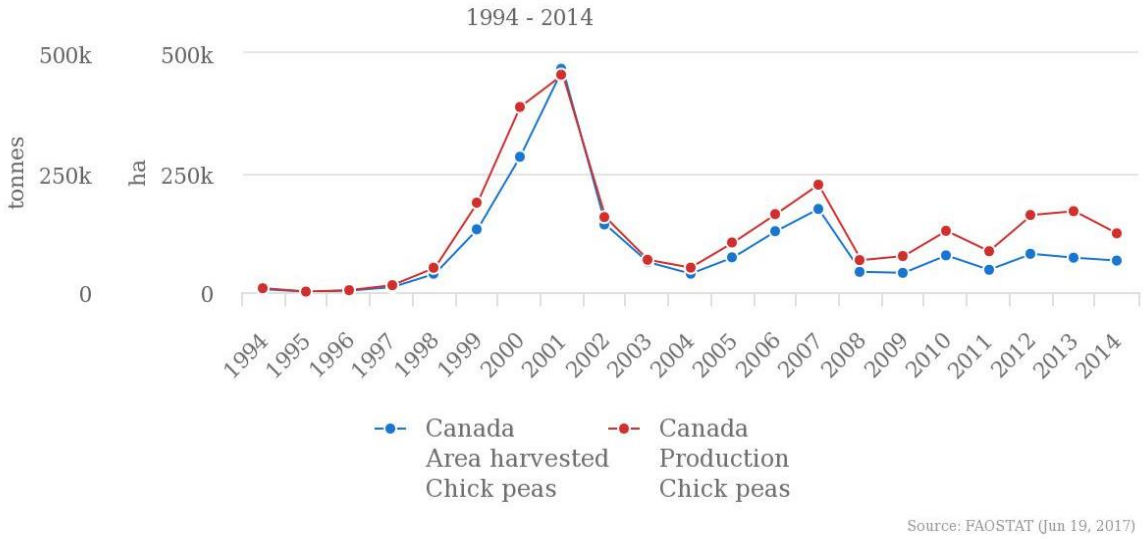

Figure 2.1. Chickpea production and area under chickpea cultivation in Canada over a twenty-year period (1994-2014; Figure source: FAOSTAT, 2017).

Figure 2.2. Chickpea growing regions (highlighted in yellow) in Canada (Figure source: Pulse Canada, 2017).

2.2 CHICKPEA GENOMIC RESOURCES

Until 2005, limited genomic resources were available for most legume crops including chickpea, but in recent years significant advancements have been made in the development of large-scale genomic resources in chickpea (Varshney et al., 2013a). In the 1990's, the first generation of Sequence Tagged Microsatellite Sites (STMS) or Simple Sequence Repeat (SSR) markers (Hüttel et al., 1999; Winter et al., 1999) and Expressed Sequence Tags (ESTs) markers were used to develop genetic maps of bi-parental chickpea populations derived from narrow and wide crosses to dissect the genetic basis of disease resistance and a few quality traits. The STMS or SSR markers were used for marker-assisted backcrossing to improve drought (Varshney et al., 2013b), fusarium wilt resistance (Varshney et al., 2014b), ascochyta blight resistance and the double podding traits (Tar'an et al., 2013) in chickpea. Tremendous progress has been achieved in the development of novel genetic tools including more molecular markers and dense genetic maps (Millan et al., 2006). Using these genomic resources, several QTLs for biotic, abiotic and agronomic traits have been identified in different mapping populations including F_2 and RIL populations (Thudi et al., 2014). Recent advances in next generation sequencing has generated large-scale transcriptome data and the second generation of genetic maps using Single Nucleotide Polymorphisms (SNPs) (Varshney et al., 2013a). The current genomic resources available in chickpea include large-scale molecular markers (approximately 2000 SSR, SNPs), high-density diversity array technology (DArT; 15360 clones), Illumina GoldenGate array, high-density comprehensive genetic maps, transcriptomes and genome assemblies of both desi and kabuli chickpeas (Varshney, 2016).

Varshney et al. (2013c) reported the draft whole genome sequence (WGS) of Canadian kabuli chickpea cultivar 'CDC Frontier' which contains 28,269 genes (http://www.icrisat.org/gt-bt/ICGGC/GenomeSequencing.htm). Another draft genome assembly of desi chickpea cultivar 'ICC4958' of 540 MB containing 27,571 genes was reported by Jain et al. (2013; http://www.nipgr.res.in/CGWR/home.php). Recently, a 416 Mb draft genome assembly of the wild progenitor of cultivated chickpea, *Cicer reticulatum* L. was reported containing 25,680 protein coding genes (Gupta et al., 2016). These genome sequences have opened a new era of genomics and molecular biology for trait improvement in chickpea including quality improvement, drought tolerance, and better resistance to diseases. New sequencing and genotyping technologies have shown their potential by allowing completion of deep sequencing and facilitating SNP discovery. Genome resequencing of 97 chickpea genotypes

generated a total of 81,845 SSRs and 48,298 SNPs which can be used for the development of a dense genetic map and detailed diversity analysis (Varshney et al., 2013c). Recently, both desi and kabuli genome assemblies have been significantly improved and updated (Edwards, 2016a, 2016b). The improved desi chickpea genome assembly covers 29,400 protein coding genes and the improved kabuli genome assembly includes 33,351 protein coding genes. The availability of these improved genome assemblies will aid in identifying candidate genes for important biological function in chickpea.

Chickpea is now a crop with sufficient resources for genomics-assisted breeding and as a result, activities including trait mapping, molecular breeding such as marker-assisted backcrossing (MABC), marker-assisted recurrent selection (MARS) and advanced backcross quantitative trait loci (AB-QTL) analysis, which were previously limited to major crops, are now routinely used in chickpea (Thudi et al., 2014). The application of genomic-assisted breeding increased the precision and efficiency in breeding for stress tolerance such as drought tolerance in chickpea (Thudi et al., 2014) and, similarly, it can be applied to other economically important traits such as disease resistance.

2.3 CHALLENGES IN CHICKPEA PRODUCTION

Under optimum growing conditions the yield potential of chickpea was predicted to be up to 6 tonnes ha^{-1}, which is much higher than the current global yield average of 0.8 tonnes ha^{-1} (Kanouni et al., 2011). Chickpea production is limited by a number of biotic and abiotic stresses that reduce yield and yield stability. The major biotic stresses in chickpea production are fungal diseases (such as ascochyta blight, fusarium wilt, botrytis grey mould) and insects (chickpea pod borer), whereas major abiotic stresses include drought, heat, cold and salinity (Millan et al., 2006). Collective annual yield losses due to abiotic stresses (6.4 million tonnes) were higher than biotic stresses (4.8 million tonnes) as estimated by Ryan (1997). Whether this trend has changed is unknown as no update has been reported recently. The severity of yield loss depends on climatic conditions and geographic location. In tropical and sub-tropical parts of the world, fusarium wilt and terminal drought are the main problems, whereas ascochyta blight, short growing season and end-of-season frost are the major constraints in temperate countries such as Canada, the USA and Australia (Pande et al., 2005).

The area of Canadian chickpea production has declined since 2002 due to ascochyta blight outbreaks (**Figure 2.1**). After some increases in production in 2012 and 2013, it has fallen again and Canadian chickpea exports fell by 53% in 2016 because of a late harvest and reduced quality from disease (Specialty Crop Report, 2016). Other than fluctuating demand for chickpea in the international markets, the major limitations in Canadian chickpea production are the short growing season and ascochyta blight.

2.4 ASCOCHYTA BLIGHT OF CHICKPEA

Ascochyta blight of chickpea is one of the most devastating plant diseases globally. The first occurrence of ascochyta blight was reported by Butler in 1911 in the north-western part of India (now Pakistan; Singh and Sharma, 2002). Since then, its occurrence has been reported in more than 40 countries with severe effects in areas where cool and humid conditions prevail during the flowering and podding stages (Sharma and Ghosh, 2016). The disease is devastating in countries where chickpea has been cultivated for many years and is rapidly spreading in countries such as Canada, the USA and Australia, where chickpea is a relatively new crop (Pande et al., 2005). Under favorable environmental conditions, i.e. relative humidity above 60% and temperatures of 10-20°C, ascochyta blight can cause up to 100% yield loss in susceptible cultivars (Reddy and Singh, 1990).

Ascochyta blight was first reported from Saskatchewan in 1974 and its introduction was thought to have occurred through imported infected seeds (Morrall and McKenzie, 1974). As the area under chickpea cultivation increased on the Canadian prairies, sporadic outbreaks of ascochyta blight causing severe yield losses were recorded in 1999-2000 (Chongo et al., 2003). Inoculum build-up, short crop rotation and the use of infested seeds were major contributors to the epidemics of ascochyta blight. The presence of favorable conditions throughout the growing season boosts disease severity in Canada. Chickpea growers in Western Canada have relied heavily on fungicide applications to manage ascochyta blight, but it is not always cost-effective. The use of resistant cultivars is considered the most economical way to manage this disease. The chickpea breeding program of the Crop Development Centre at the University of Saskatchewan has developed and commercialized new chickpea cultivars with improved resistance to ascochyta blight in the past decade (Warkentin et al., 2005). Currently, most chickpea cultivars are moderately resistant to

ascochyta blight and require only 2-3 fungicide applications (Saskatchewan Pulse Growers, 2017).

2.4.1 CAUSAL ORGANISM

Ascochyta blight of chickpea is caused by the ascomycete fungus *Ascochyta rabiei* (Pass.) Labrousse (Pande et al., 2005; Sharma and Ghosh, 2016). This pathogen has two stages in its life cycle: asexual (anamorph) and sexual (teleomorph). The characteristic feature of the anamorph, *A. rabiei*, is the formation of dark spherical fruiting bodies called pycnidia. Numerous asexual conidia or pycnidiospores are produced and differentiated on conidiophores in a mucilaginous mass in the pycnidia. The teleomorph, *Didymella rabiei* (Kovacheski) var. Arx is a bi-polar heterothallic fungus with the two mating types MAT1-1 and MAT1-2. The characteristic feature of the sexual form is the development of dark brown to black colored pseudothecia on over-wintering crop debris. Ascospores are produced by mating of two compatible mating types which ensures sexual recombination resulting in genetic diversity (Pande et al., 2005). Both pycnidia and pseudothecia can develop on over-wintering infested chickpea debris (Kanouni et al., 2011).

Recently the draft genome sequence of an Indian *A. rabiei* isolate (ITCC No. 4638) identified it as mating type 2 (MAT1-2) with total genome assembly size of 34.6 Mb (Verma et al., 2016). The *A. rabiei* genome assembly predicted 10,596 protein-coding genes which include secretory proteins, transporters and primary and secondary metabolism enzymes reflecting the necrotrophic nature of *A. rabiei*. The comparative genome analysis showed the closeness of the *A. rabiei* genome to the wheat necrotrophic pathogen *Stagonospora nodorum*. Total predicted protein coding genes in *A. rabiei* were comparatively fewer than those of the necrotrophic fungal pathogens *Pyrenophora tritici-repentis* (12,141) and the hemibiotrophic *Cochliobolus sativus* (12,250) (Verma et al., 2016).

2.4.2 SYMPTOMS

Following *A. rabiei* infection, symptoms in the form of round and elongated brown necrotic lesions spread rapidly on all aerial parts of the plant including leaves, petioles, flowers, pods, stems and branches leading to collapse of tissue resulting in plant death (Nene, 1982; Pande et al., 2005; Sharma and Ghosh, 2016). Black colored pycnidia in concentric rings on leaves and pods are the typical symptom of *A. rabiei* infection which results in leaf drop and infected pods. Lesions that develop on stems and branches are elongate in shape, and as these lesions enlarge in size they can girdle the stem causing breakage and plant parts above the

stem lesion die rapidly. Depending on inoculum pressure, plants can be attacked at any growth stage, but flowering to the early podding stage is a most prominent stage for infection. Infection at the early podding stage results in diseased pods, which often fail to produce any seed. Infection at the pod maturation stage often results in seed infection through testa and cotyledon resulting in shriveled and discolored seeds (Pande et al., 2005).

The disease symptoms in the field initially appear in small patches of blighted plants (Pande et al., 2005). In cases where the source of inoculum is infected seeds uniform symptoms can be seen across the field (Kanouni et al., 2011). Under cool moist conditions, the disease spreads rapidly throughout the field within days and weeks.

2.4.3 DISEASE EPIDEMIOLOGY

Ascochyta blight is a seed-borne disease and spreads through air-borne ascospores and water-splashed conidia (Nene, 1982). The primary sources of infection are infested seeds and crop debris (Figure 2.3; Kaiser, 1997). Infected seeds result in a uniform distribution of infected seedlings which act as primary sources of infection (Gan et al., 2007). Infected seed transmission is responsible for the introduction of ascochyta blight into countries such as Canada, the USA, Iran, and Australia (Kaiser, 1997). During the off-season, *A. rabiei* survives on crop debris as asexual pycnidia (Navas-cortes et al., 1995) or at various stages of teleomorphic development (Kaiser, 1997; Trapero-Casas and Kaiser, 1992). Conidia produced from these pycnidia serve as primary inoculum for the next season. In regions where the teleomorph occurs, the primary infection starts with ascospores produced from infected debris. Ascospores are important in long distance dispersal of the pathogen and may spread as far as 100-400 m from the inoculum source by wind (Pande et al., 2005; Trapero-Casas et al., 1996). Secondary spread of the disease is through the dispersal of conidia and ascospores by wind, water and rain splash (Pande et al., 2005). Environmental conditions play an important role in ascochyta blight development and severity. Low temperature and high moisture are necessary for disease initiation (Pande et al., 2005). Disease development occur at a temperature range of 5-30°C with an optimum of 20°C, and 17 hours of wetness are essential to produce severe infection. Cloudiness or periods of darkness after inoculation also increases disease severity.

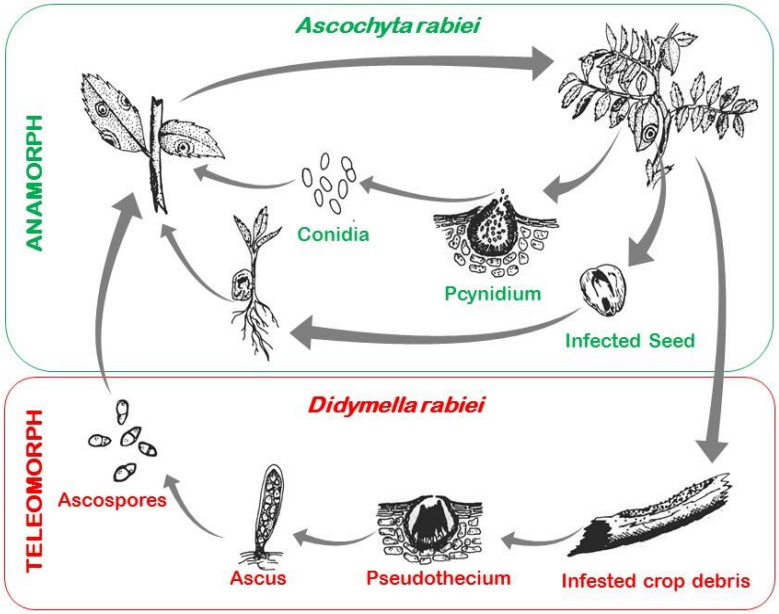

Figure 2.3. Lifecycle of *Ascochyta rabiei* (Figure modified from Kaiser, 1997).

2.4.4 HOST RANGE OF *ASCOCHYTA RABIEI*

The review by Pande et al. (2005) suggested that *A. rabiei* has a broad host range as artificial inoculation on lentil, field pea, vetch, common bean, and cowpea showed that the fungus is pathogenic to all of these species. *A. rabiei* infects several other plant species that grow in chickpea growing areas such as *Vigna unguiculata*, *Phaseolus vulgaris*, *Lactuca serriola*, *Lamium amplexicaule*, *Medicago sativa*, *Melilotus alba* and *Thlaspi arvense*. In contrast, Peever (2007) suggested that *A. rabiei* is host-specific to chickpea based on the study of Hernandez-Bello et al. (2006) showing that artificial inoculation of *A. rabiei* on lentil, vetch and pea were unsuccessful and all three plant species remained free from infection. As a result, host specificity of *A. rabiei* remains unclear.

2.4.5 MODE OF INFECTION

After the successful landing of spores (ascospores or conidia) on chickpea leaves, spores begin to swell and germinate within 12 hours to develop germ tubes which elongate on the leaf surface (Pandey et al., 1987). The germ tubes secrete mucilaginous exudates which act as an adhesive on the plant surface protecting the germ tube from desiccation and sealing the infection site (Jayakumar et al., 2005). Penetration normally occurs 24 hours post inoculation through the leaf cuticle, stem cuticle and stomatal openings (Kanouni et al., 2011; Pandey et

al., 1987). As hyphal branches form non-melanized appressorium-like structures, penetration of the cuticle through mechanical forces is not likely, but instead is accompanied by cell wall degrading enzymes such as cutinase, xylanase and pectinase (Jayakumar et al., 2005). Being a necrotrophic fungus, *A. rabiei* produce toxins after successful penetration into the host. These toxins are responsible for necrosis of the host tissue during the infection process. The pathogen produces three different types of phytotoxins: Solanapyrones (A, B and C), cytochalasin D, and a proteinaceous phytotoxin. These phytotoxins are presumed to be important factors in host-pathogen interaction (Jayakumar et al., 2005).

In the initial stage of infection, hyphae grow parallel between epidermal and palisade parenchyma cells and spread within the intercellular space. It usually takes four days post inoculation (dpi) for necrotic lesions to appear on chickpea leaves (Kohler et al., 1995). In the later stage of infection (6 dpi), the entire cortex and pith are disintegrated. Pycnidia start to mature on infected leaves and pods, and become visible as black dots (Pandey et al., 1987). Lignified tissues are slightly damaged, whereas non-lignified tissues are completely destroyed resulting in the girdling and collapse of plants (Pande et al., 2005). By the end of infection, all cells are destroyed and filled with fungal biomass.

2.4.6 PATHOGEN VARIABILITY

The presence of a teleomorph stage in the *A. rabiei* life cycle results in sexual recombination which contributes to variability within the pathogen populations. The sexual recombination may generate a new combinations of virulence genes and development of new pathotypes. Thus, *A. rabiei* shows a high degree of pathogenic and genetic variability (Sharma and Ghosh, 2016). Several pathotypes of *A. rabiei* of chickpea were reported, e.g. more than ten pathotypes by Vir and Grewal (1975), five pathotypes by Nene and Reddy (1987), ten pathotypes by Ali et al. (2009), and three pathotypes by Udupa et al. (1998). More recently a new *A. rabiei* pathotype (pathotype IV) was identified in Syria that is capable of causing disease on chickpea genotypes highly resistant to all three pathotypes described by Udupa et al. (1998) (Imtiaz et al., 2011). In India, four distinct population groups were reported from 64 *A. rabiei* isolates collected from different agroclimatic regions using AFLP and SSR markers (Varshney et al., 2009) and 10 *A. rabiei* pathotypes were reported based on their morphological variation by Kaur et al. (2012).

2.4.7 POPULATION STRUCTURE OF *ASCOCHYTA RABIEI* IN CANADA

A. rabiei was introduced to Canada with the movement of infected chickpea seeds (Morrall and McKenzie, 1974). Both mating types of the pathogen were identified and the teleomorph of *A. rabiei* was first reported in 1999 from Western Canada (Armstrong et al., 2001). Based on RAPD markers, 14 pathotypes with high levels of genetic diversity due to sexual recombination were reported among 40 isolates of *A. rabiei* collected in 1998-1999 in Canada (Chongo et al., 2004). In another study using 99 isolates of *A. rabiei* collected in 2001 and 2002 from the Canadian prairies and seven differential genotypes, a shift in the population towards higher aggressiveness was observed, but no race or pathotype structure was observed (Vail and Banniza, 2008). In the USA, the population of *A. rabiei* was classified into two broad categories: less aggressive as pathotype I and more aggressive as pathotype II (Chen et al., 2004). To understand the genetics of virulence in *A. rabiei*, a study was conducted by Peever et al. (2012). In this study, a genetic cross was made between a highly virulent isolate 'AR628' from Syria and less virulent isolate 'AR20' from the USA which were previously classified as Pathotype I and II, respectively. From this cross 77 independent progeny isolates were used for inoculating susceptible and resistant chickpea genotypes and revealed that there is a continuum of aggressiveness among isolates rather than discrete pathogenic groups. These results indicated that virulence is a quantitative character and controlled by more than one genetic locus.

2.4.8 DISEASE MANAGEMENT

Ascochyta blight damage in chickpea can be minimized by following agronomic or cultural practices, using chemical control and using resistant cultivars. The integrated approach using all three measures produce an optimum disease management strategy as each individual measure is not completely effective (Gan et al., 2006). The cultural practices include the use of ascochyta blight-free seeds, field sanitation, and sowing strategies. As this disease spreads through infected seed and stubble-borne inoculum, cultural practices such as the use of ascochyta blight-free seeds will prevent the disease transmission to seedling. *A. rabiei* can survive for more than two years on crop debris if left on the soil, whereas burying the infected stubble under the soil at the depth of 10-20 cm can shorten the survival of *A. rabiei* using conventional tillage practices (Gan et al., 2006). A minimum of three to four-year crop rotation with non-host crops reduces inoculation potential from crop stubble.

Sowing time is critical for ascochyta blight infection, as chickpea can be sown either in winter or spring, depending on geographic location (Gan et al., 2006). Studies conducted at ICARDA over a 10-year period in Syria and Lebanon have shown that winter-sown chickpeas are higher yielding than the spring-sown, but the damage due to disease is greater on winter-sown than spring-sown chickpeas due to cool and humid conditions. Early or delayed sowing is advised to avoid the exposure of plants to the pathogen during the period of maximum abundance of spores or cool and wet condition at flowering stage. However, in Western Canada, regardless of ascochyta blight the sowing cannot be delayed due to the short growing season.

Chemical control for ascochyta blight includes seed coating with fungicides (seed dresssing) and foliar applications. Seed dressing prevent seed-borne infection as they limit spore germination and mycelial growth on the seed surface. Ascochyta blight can reach epidemic levels very quickly, thus foliar fungicide application is often required for partially resistant cultivars under conditions conducive to disease development (Gan et al., 2006). Numerous fungicides have proven effective against ascochyta blight and under epidemic conditions multiple fungicide applications are required. This practice, however, may increase the probability of fungicide-resistance in the fungus, and may also be uneconomical.

2.5 GENETIC BASIS OF ASCOCHYTA BLIGHT RESISTANCE IN CHICKPEA

Numerous studies have reported varying results related to the number of genes controlling ascochyta blight resistance in chickpea. Initially, it was reported that the resistance was controlled by two dominant complementary genes (Ahmad et al., 1952). It was reported that a single dominant gene govern resistance against ascochyta blight in desi chickpea (Vir et al., 1975). In kabuli chickpea, a single dominant and a single recessive gene governed the resistance against ascochyta blight depending on the parent (Singh and Reddy, 1983; Tewari and Pandey, 1986), whereas two complementary dominant genes (Dey and Singh, 1993) and three major genes with complementary effects and other minor genes were also reported to be responsible for resistance in chickpea germplasm (Tekeoglu et al., 2000).

Resistance to ascochyta blight is now considered a quantitative trait and over the last decade, many attempts have been made to map ascochyta blight resistance QTLs using various molecular markers and different genetic backgrounds. Santra et al. (2000) identified two QTLs, *QTL-1* and *QTL-2* conferring resistance to ascochyta blight in a RIL population

derived from a cross between 'ILC 3279/FLIP 84-92C' that accounted for 50% and 45% of the total phenotypic variation for two subsequent years, respectively. *QTL-1* was tagged with Random Amplified Polymorphic DNA (RAPD) markers on LG 6 and *QTL-2* was tagged with Inter-Simple Sequence Repeat (ISSR) markers on LG 1. Using the same RIL population, Tekeoglu et al. (2002) further demonstrated that *QTL1* is closely linked to GAA47, a Simple Sequence Repeat (SSR) marker, and ubc733, an isozyme marker, whereas *QTL 2* is associated with TA72, TA2, TS54, and TA146 SSR markers. Flandez-Galvez et al. (2003) reported that genomic regions on LG 1, LG 2 and LG 3 are associated with resistance to ascochyta blight in a desi chickpea population derived from a cross involving 'ICC 12004' as a resistance source and 'Lasseter' as a susceptible source under both field and controlled conditions. Cobos et al. (2006) reported a major QTL on LG 2 accounting for 28% of the phenotypic variation using an interspecific RIL population derived from a cross between cultivated resistant cultivar 'ILC 72' (*C. arietinum* L.) and the susceptible wild germplasm accession, 'Cr5-10' (*C. reticulatum*). Lichtenzveig et al. (2006) reported three major QTLs, two QTLs on LG 4 and one QTL on LG 8 using SSR markers in a RIL population derived from a cross between a kabuli cultivar and a desi accession. Tar'an et al. (2007) identified one QTL each on LG 3 explaining 16% of phenotypic variation, on LG 4 explaining 29% of phenotypic variation, and on LG 6 explaining 12% of phenotypic variation. The QTL on LG 3 was unique to the population derived from an intraspecific cross involving 'ICCV96029/CDC Frontier'. Anbessa et al. (2009) reported 5 QTLs, on LG 2, 3, 4, 6 and 8, in four F_2 populations derived from crosses between the four resistant cultivars 'CDC Frontier', 'CDC Luna', 'CDC Corinne' and 'Amit' with the susceptible cultivar 'ICCV 96029'. Altogether these QTLs explained 56%, 48%, 38% and 14% of the estimated phenotypic variation in four F_2 populations, respectively. Taleei et al. (2009) found three QTLs in $F_{2:3}$ population derived from a cross between resistant cultivar 'ICC 12004' and susceptible local variety 'Bavanij', one QTL each on LG 3 (11%), LG 4 (17%) and LG 6 (19%) which together accounted for 46.5% of total phenotypic variation. Aryamanesh et al. (2010) found three QTLs in an interspecific F_2 population, one QTL on LG 3 and two QTLs on LG 4 which explained 49% of phenotypic variation for resistance to ascochyta blight. Sabbavarapu et al. (2013) reported 6 QTLs (three QTLs on LG 4, one QTL on LG 5 and two QTLs on LG 6) explaining 32% of phenotypic variation under field and greenhouse conditions. Stephens et al. (2014) reported two QTLs using SNP markers in two populations, one QTL was common with previous studies and the other QTL was novel. Recently, Daba et al. (2016) identified 8 QTLs on each linkage group, except LG 5, for ascochyta blight

resistance that explained 10 to 19% of phenotypic variations using SNP markers in the RIL population advanced from an F_2 population used by Tar'an et al. (2007).

2.6 CHALLENGES IN BREEDING FOR ASCOCHYTA BLIGHT RESISTANCE

Breeding for resistance to ascochyta blight is one of the most important objectives of chickpea breeding programs in North America, Australia, Europe and some parts of Middle-East. The efforts to breed for ascochyta blight resistance were started in the early 1940's (Luthra et al., 1935), but success in developing chickpea varieties with improved levels of resistance has been limited due to the complex nature of the resistance (Rubiales and Fondevilla, 2012). Chickpea germplasm is well known to have very limited genetic diversity due to a series of evolutionary bottlenecks (Abbo et al., 2003). The major challenge in breeding for ascochyta blight resistance is the lack of high level of resistance to ascochyta blight in the primary gene pool of chickpea. Singh and Reddy (1993) reported that among 19,343 chickpea accessions only five accessions (ICC4475, ICC6328, ICC12004, ILC200, and ILC6428) showed stable resistance to six races of *A. rabiei* in Syria. Few other source of resistance such as ICC3996 were identified (Chen et al., 2004). Most of the sources of resistance to ascochyta blight in the primary gene pool can, however, only provide incomplete protection (Sharma and Ghosh, 2016).

Another factor is the breakdown of resistance in previously known source of resistance due to the sexual recombination, mutation in pathogen population and the emergence of new pathotypes (Sharma and Ghosh, 2016). In Syria, a new *A. rabiei* pathotype (pathotype IV) was identified that can cause disease on chickpea genotypes ICC12004 and ICC3996 previously resistant to three *A. rabiei* pathotypes (Imtiaz et al., 2011).

Using partially resistant genetic germplasm, numerous studies have reported varying results on the inheritance of resistance to ascochyta blight as both a monogenic (Vir et al., 1975; Singh and Reddy, 1983; Tewari and Pandey, 1986) and polygenic trait (Tekeoglu et al., 2000). Using conventional breeding approaches, several QTLs have been identified on all linkage groups of the chickpea genome (Anbessa et al., 2009; Aryamanesh et al., 2010; Cho et al., 2004; Cobos et al., 2006; Daba et al., 2016; Flandez-Galvez et al., 2003; Iruela et al., 2006; Lichtenzveig et al., 2006; Madrid et al., 2014; Sabbavarapu et al., 2013; Santra et al., 2000; Stephens et al., 2014; Tar'an et al., 2007; Udupa and Baum, 2003). The detection of ascochyta blight resistance QTLs on all eight chickpea linkage groups indicates that the resistance is governed by multiple QTLs or genes which are distributed across the genome.

Pyramiding different sources of resistance within cultivated chickpea species carrying different genes and QTLs can improve the levels of resistance and may provide durable resistance to ascochyta blight in chickpea (Anbessa et al., 2009). Partial resistance is more durable due to the action of multiple loci in contrast to complete resistance which is governed by plant disease resistance genes and is very often less durable due to the continuous evolution of the pathogen (Marone et al., 2013). Despite the abundance of identified QTLs for ascochyta blight, success in introgression of known QTLs has been limited due to the large size of the QTL regions (up to 30 Mb) and disassociation of the linked markers from the resistance locus through recombination (Li et al., 2017; Rubiales and Fondevilla, 2012).

2.7 CANDIDATE GENES IDENTIFIED FOR ASCOCHYTA BLIGHT RESISTANCE

Considerable progress has been made using conventional breeding approaches to improve chickpea cultivars for resistance to ascochyta blight (Pande et al., 2005). Identification of candidate genes involved in resistance to ascochyta blight will help in understanding the resistance mechanism and will further assist in the development of resistant cultivars using marker-assisted selection. The availability of the draft genome sequence of chickpea provided the resource to identify the genomic regions and candidate genes across the genome (Gupta et al., 2016; Jain et al., 2013; Varshney et al., 2013c). Integration of the physical map and two genetic maps using 245 bacterial end sequence derived simple sequence repeat markers assisted in identifying 306 candidate genes present in the vicinity of ascochyta blight resistance QTLs (Varshney et al., 2014a). Two candidate genes, *CaETR1* (*EIN-4 like*) and *ethylene insensitive 3-like gene* (*Ein3*) from the ethylene pathway, were identified in ascochyta blight resistance QTL_{AR1} on LG 4 and QTL_{AR3} on LG 2, respectively (Madrid et al., 2012, 2014). These studies suggested the possible involvement of the ethylene pathway in the ascochyta blight resistance in chickpea. Few expression studies have been conducted to identify candidate genes for ascochyta blight resistance in chickpea. Transcriptome profiling using 756 microarray identified 97 differentially expressed candidate genes upon *A. rabiei* infection (Coram and Pang, 2006). In a recent study, expression profiling of 15 defence-related genes in response to *A. rabiei* infection identified six differentially expressed genes among ten chickpea genotypes (Leo et al., 2016). The most recent study identified a novel candidate gene from the Receptor-Like Kinase (RLK) class of R-genes using WGS and GWAS (Li et al., 2017). The study by Li et al. (2017) identified a 100 kb region (AB4.1) on

chromosome 4 associated with ascochyta blight resistance and this region was co-located with a previously reported QTL interval identified in three different mapping populations. In total, 12 predicted genes were located in the AB4.1 region but only one significant SNP was identified in the catalytic domain of the RLK gene and further transcriptional analysis showed that this gene was significantly induced in resistant lines after inoculation in comparison to non-inoculated plants.

2.8 PLANT IMMUNE SYSTEM

Plants and pathogens have co-evolved together and each has developed different survival strategies. Plant pathogens have gained the ability to invade plants, suppress plant defense responses and colonize plant tissue for their growth and reproduction. To cope with the wide array of pathogens, plants have developed a sophisticated immune system (Hammond-Kosack and Jones, 1997; Qi and Innes, 2013). The plant immune system differs from that of vertebrate animals in that it is not adaptive. Instead, plants solely rely on a bi-layered cell-autonomous immune system to perceive and respond to the invading pathogens (Jones and Dangl, 2006).

Most pathogen infections are prevented by the first layer of plant basal defence response which provides broad and non-host resistance upon recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) by the pattern recognition receptors (PRRs) localized on the plasma membrane. This is called PAMP-triggered immunity (PTI) (Jones and Dangl, 2006). PAMPs are highly conserved signature characteristic of microbes such as fungal chitin and bacterial flagellin. Plant PRRs are either plasma membrane-localized receptor-like kinases (RLKs) or receptor-like proteins (RLPs) (Zipfel, 2014). RLKs contain a ligand-binding ectodomain, a single-pass transmembrane domain, and an intracellular kinase domain, whereas RLPs are similar in structure but lack the intracellular kinase domain involved in signaling (Boutrot and Zipfel, 2017). Activation of PTI results in changes in ion fluxes across the plasma membrane, rapid production of reactive oxygen species, expression of defense related genes, reinforcement of cell walls and induced systemic acquired resistance (Glazebrook et al., 1997). However, some specifically adapted pathogens can overcome the first barrier by delivering effector proteins into plant cells to suppress the host basal defense (Jones and Dangl, 2006). Such host-specific pathogens are countered by the second layer of defense, termed effector-triggered immunity (ETI), mediated by the intracellular receptors encoded by the R-genes that recognize the

presence of pathogen effector protein directly or indirectly and activate downstream immune responses to limit pathogen growth. The activation of ETI leads to a similar, but a more rapid and extreme version of a PTI defense response, typically resulting in programmed cell death.

2.9 NBS-LRR GENES

R-genes are the key component of the plant immune system to recognize and respond to a wide range of pathogens. R-genes are diverse in terms of structure, function and evolution. Several classes of R-genes have been identified and classified based on their putative protein domain organization and their localization in the plant cell (Ellis et al., 1999). Most cloned R-genes so far encode proteins with a central Nucleotide Binding Site (NBS) domain and a carboxyl/C-terminal Leucine Rich Repeat (LRR) domain, hence are called NBS-LRR genes (McHale et al., 2006). Homologs of NBS-LRR genes have been identified in *A. thaliana* (Meyers et al., 2003), *Oryza sativa* (Monosi et al., 2004), *Medicago truncatula* (Ameline-Torregrosa et al., 2008), *Manihot esculenta* (Lozano et al., 2015), *Glycine max* (Kang et al., 2012), *Brassica rapa* (Mun et al., 2009), *Solanum tuberosum* (Lozano et al., 2012) and in many other plant species. Most studies identified variable numbers of NBS-LRR genes ranging from 50 in *Carica papaya* (Porter et al., 2009) to 1,015 in *Malus domestica* (Arya et al., 2014). The NBS-LRR genes are unevenly distributed in plant genomes and are often found in clusters (Meyers et al., 2003).

The presence of an NBS domain places the NBS-LRRs into a separate sub-class of the signal transduction ATPases with numerous domains (STAND) family of proteins, a class of molecular switches involved in processes such as immunity, apoptosis and transcriptional regulations (Takken and Goverse, 2012). Plant NBS-LRR proteins mediate ETI, as NBS-LRR proteins are the intracellular receptors that recognize the presence of pathogen effectors directly by binding to the pathogen effector proteins, or indirectly by recognition of any modification in the pathogen effector target proteins in the host. Upon recognition, they activate multiple defence signal transduction pathways which often result in a hypersensitive response and other biochemical changes that limit pathogen growth (DeYoung and Innes, 2006; Meyers et al., 2003).

Plant NBS-LRR genes can be classified into two sub-classes based on the presence or absence of an amino/N-terminal domain. The first sub-class comprises of proteins that carry *Drosophila* Toll and INTERLEUKIN1 like receptor (TIR) domains at the N-terminal position

and are called TIR-NBS-LRR (TNL). The other sub-class comprises proteins which often carry a Coiled-Coil (CC) domain and are known as CC-NBS-LRR (CNL; Meyers et al., 1999). Other domains such as a Zinc Finger or RPW8 domains are also found at the N-terminal position instead of the CC domain and are often classified as the CNL class (Sukarta et al., 2016). The distribution of TNL and CNL gene classes is species-specific as dicots contain both classes while monocots lack the TNL class (Shao et al., 2016).

NBS-LRR genes encode the largest proteins known in plants ranging from 860 to 1,900 amino acids, which contain characteristic structural features conserved in many plant species (McHale et al., 2006). At least four distinct domains are present: a variable amino-terminal domain, the NBS domain, the LRR domain, and variable carboxyl-terminal domains joined by linker regions which together form a signaling competent immune receptor (McHale et al., 2006; Sukarta et al., 2016).

2.9.1 AMINO-TERMINAL DOMAIN

The variable amino or N-terminus of NBS-LRR genes may consist of a TIR or CC domain (McHale et al., 2006). Significant progress has been made in resolving the structure of N-terminal domains (Sukarta et al., 2016). Recent evidence indicates that these domains have a strategic role in pathogen recognition and signaling (Chang et al., 2013; Williams et al., 2014). The TIR domain identified in both plant and animals consists of three conserved motifs (Slack et al., 2000). The TIR domain is thought to function as an adaptor domain involved in protein-protein interactions (Sukarta et al., 2016). Often these interactions involve self-associations or homotypic interactions with other TIR domains. In animals, the TIR domain is involved in signaling downstream of Toll-like receptors. The role of the CC domain is poorly understood and controversial. For example, over-expression of the CC domain alone in *Nicotiana benthamiana* can induce cell death while the CC domain of the barley MLA protein interacts with WRKY transcription factors in the nucleus which inhibit their ability to repress defense genes (Qi et al. 2012).

2.9.2 NBS DOMAIN

The central NBS also known as NB-ARC (nucleotide binding adaptor shared by NOD-LRR proteins, Apaf1, R-proteins and CED4) domain found in plant NBS-LRRs and several metazoan apoptosis regulators consist of three structural sub-units: the NB, ARC1 and ARC2. Together form nucleotide-binding pocket (Sukarta et al., 2016; Takken and Goverse, 2012). The NBS domain consists of several conserved motifs in strict order, including P-loop/Kinase

1a, RNBS-A, Kinase2, RNBS-B, RNBS-C, GLPL, RNBS-D, and MHDV. The sequence of the three resistance NBS (RNBS) motifs, RNBS-A, RNBS-C and RNBS-D can distinguish the NBS domain of TNLs and CNLs (Meyers et al., 2003). The crystal structure of plant NB-ARC is not available yet, so the structurally related Apaf1, CED4, and several other STAND ATPase have been used as a template for homology modeling (Takken and Goverse, 2012). Remote homology modeling revealed a compact globular structure for the NB-ARC domain.

The general function of NBS-LRR proteins is thought to be that of a molecular switch, in which the NB-ARC domain is the core of the molecular switch (Qi and Innes, 2013; Sukarta et al., 2016). The Adenosine diphosphate (ADP)-bound is considered the 'off' state and during this state NB-ARC adopts a closed structure forming a nucleotide-binding pocket which encloses a bound ADP (Takken and Tameling, 2009). Activation is thought to require the release of ADP and to be replaced by Adenosine triphosphate (ATP). The ATP-bound configuration is considered the 'on' state, which requires the partial opening of the closed nucleotide-binding structure to allow for an exchange of ADP with ATP. Hydrolysis of ATP returns the protein to the off state.

2.9.3 LRR DOMAIN

The LRR domains present in the C-terminus of the central NBS domain, characterized by a repetitive sequence pattern rich in hydrophobic leucine residues alternating with hydrophilic residues in a fixed pattern LxxLxLxxN/CxL, where x can be any amino acid and L positions can also be occupied by valine, isoleucine and phenylalanine (Kobe and Kajava, 2001; Sukarta et al., 2016). The LRR domain is a versatile structural motif that has been identified in thousands of proteins in all life forms (Bella et al., 2008). The LRR domain is found in a functionally diverse array of intracellular, extracellular and membrane-attached proteins with the common thread of being involved in protein-protein interactions. LRRs domains are typically repeats of 20-30 amino acids and are built from tandems of two or more repeats. The LRRs in plant NBS-LRRs are highly irregular with varying repeat lengths and numbers of repeats (Takken and Goverse, 2012). In *A. thaliana* the LRR domains of TNLs and CNLs contains a similar number of repeats (8-25, mean 14; Meyers et al., 2003).

The crystal structure of LRR domains was first deduced from the porcine ribonuclease inhibitor (RI) (Kobe and Deisenhofer, 1993). Since then, nearly 90 structures of LRR proteins have been established. (Bella et al., 2008). Typically, individual LRRs consist of β-strands and α-helix-loop units, and the repeats are arranged such that the structure forms a horseshoe-

shaped or curved solenoid structure where each repeat is a turn of the solenoid. The hydrophobic leucine residues face inwards and tightly pack together to form a stable hydrophilic core, whereas the hydrophilic residues are exposed and compose a binding surface for protein-protein interaction. In NBS-LRR proteins, putatively solvent exposed residues in β-sheets may interact with pathogen ligands which determine specificity for pathogen elicitors and have shown to be often under diversifying selection (Mondragon-Palomino, 2002).

The LRR domain is thought to play a dual role in R-protein function: autoinhibition via intramolecular interactions with the NB-ARC or N-terminal domains, and activation of defense responses upon pathogen recognition (Takken and Tameling, 2009). The N-terminal of the LRR is thought to act in negative regulation, as mutation in the N-terminal LRR domain of *Rx*, a CNL class gene that confers resistance to Potato virus X through recognition of the viral coat protein, causes slight auto-activation. However to induce a strong HR response, co-expression of both LRR halves is required, therefore the LRR domain also provides positive control (Lukasik and Takken, 2009). The C-terminal of the LRR domain is thought to be involved in pathogen recognition specificity based on the domain swap experiments between *Rx* and its paralogue *Gpa2*.

CHAPTER 3

GENETIC ANALYSIS OF THE NBS-LRR GENE FAMILY IN CHICKPEA AND GENE EXPRESSION PROFILES IN RESPONSE TO ASCOCHYTA BLIGHT INFECTION

CHAPTER 3: GENETIC ANALYSIS OF THE NBS-LRR GENE FAMILY IN CHICKPEA AND GENE EXPRESSION PROFILES IN RESPONSE TO ASCOCHYTA BLIGHT INFECTION.

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3.1 INTRODUCTION

In the past two decades, more than 100 R-genes conferring resistance to diverse pathogens have been cloned and characterized from different dicotyledonous and monocotyledonous plant species (Gururani et al., 2012). Most cloned R-genes encode a limited set of proteins with conserved domains even though they govern resistance towards diverse pathogens (Jones and Dangl, 2006). Based on the presence or absence of these domains, the R-gene family is divided into five major classes (Hammond-Kosack, K.E. and Jones, 1997). The first class of R-genes encodes a serine/threonine protein kinase and lacks a leucine rich repeat (LRR) domain. Receptor-like protein (RLP) is the second class of R-genes, which encode extracellular LRR domains and transmembrane domains. The third class of R-genes is characterized by receptor-like kinase (RLK) with an extracellular LRR domain, a transmembrane domain, and an intracellular kinase domain. The fourth class of R-genes contains genes encoding proteins with a nucleotide binding site (NBS) and LRR domain (Young, 2000). The fifth class of R-genes consists of all other genes which cannot be classified into these four classes due to their unique structures.

The NBS-LRR gene class is the largest R-gene class which shares structural and functional homology with the mammalian nucleotide-binding oligomerization domain (NOD)-LRR gene class. NOD-LRR containing genes are involved in immune responses and function in inflammatory responses (DeYoung and Innes, 2006; Jones and Dangl, 2006). Plant NBS-LRR proteins are mainly intracellular receptors that can perceive the presence of pathogen effectors directly by binding to pathogen effector proteins, or indirectly by recognition of any

modification in the pathogen effector target proteins in the host. Effector perception results in the activation of multiple defense signal transduction pathways which often culminate in the hypersensitive response and other biochemical changes that limit pathogen growth (DeYoung and Innes, 2006; Meyers et al., 2003).

Based on the presence or absence of an N-terminal domain, the NBS-LRR genes are classified into two sub-classes. The first sub-class comprises proteins that possess the *Drosophila* Toll and INTERLEUKIN1 like receptor (TIR) domain at the N-terminal position and are referred to as the TIR-NBS-LRRs (TNLs). The other sub-class comprises proteins which carry a Coiled-Coil (CC) domain at the N-terminal position and are known as CC-NBS-LRRs (CNLs; Meyers et al., 1999). Other than TIR or CC, domains such as Zinc Fingers or RPW8 are also found in the N-terminal position and are often classified under the CNL class (Sukarta et al., 2016). The distribution of the TNL and CNL classes is species specific. Dicots contain both classes, while monocots lack the TNL class (Shao et al., 2016). The availability of the genome sequence of desi, kabuli and wild chickpea (Gupta et al., 2016; Jain et al., 2013; Varshney et al., 2013c) has provided an opportunity to explore the genome-wide distribution of several gene families such as the Aux/IAA gene family (Singh and Jain 2015), F-box genes (Gupta et al., 2015), ERF genes (Deokar et al., 2015), CaNAC genes (Ha et al., 2014), UDP-glycosyltransferase genes (Sharma et al., 2014) and many others. Recently, the genome assemblies of both desi and kabuli chickpeas were significantly improved and updated (Edwards, 2016a, 2016b). Genome-wide analysis of the NBS-LRR gene family has been conducted in species with available genome sequence such as *A. thaliana* (Meyers et al., 2003), *Oryza sativa* (Monosi et al., 2004), *Medicago truncatula* (Ameline-Torregrosa et al., 2008), *Manihot esculenta* (Lozano et al., 2015), *Glycine max* (Kang et al., 2012), *Brassica rapa* (Mun et al., 2009), and *Solanum tuberosum* (Lozano et al., 2012). Due to its importance in the plant innate immune system, this gene family has been the target for analysis of candidate disease resistance genes. Considering the critical role of NBS-LRR genes in the plant defense system against multiple pathogens, it is important to explore the NBS-LRR gene family in chickpea and examine their involvement against ascochyta blight infection.

The objective of this study was to first identify the homologs of NBS-LRR genes and examine their structural diversity, conserved domain architecture and genomic distribution in the chickpea genome. The second objective of this study was to determine the association and co-localization of the NBS-LRR genes with the previously reported QTLs for ascochyta

blight resistance and examine the response of NBS-LRR genes upon ascochyta blight infection in chickpea.

3.2 MATERIAL AND METHODS

3.2.1 IDENTIFICATION AND CLASSIFICATION OF CHICKPEA NBS-LRR GENES

To identify the NBS-LRR genes in the chickpea genome, the genome assembly of 'CDC Frontier' including the predicted gene model annotation was downloaded from NCBI (http://www.ncbi.nlm.nih.gov/assembly/GCF_000331145.1). Predicted protein sequences of 28,269 genes in the chickpea genome were initially scanned for the Hidden Markov Model (HMM) profile of the NBS/NB-ARC domain (pfam00931) in HMMER v3.1b2 using "hmmsearch" with an expected value (e-value) threshold of 1e-04. The presence of the NBS domain was further confirmed with the NCBI conserved domain database (CDD) tool using an e-value of 0.01 (Marchler-Bauer et al., 2011). The CDD results also confirmed the presence or absence of additional domains such as TIR, CC, and RPW8 in the N-terminal position and a variable number of LRR domains in the C-terminal position. The chickpea NBS-LRR genes were classified based on their protein domain arrangements. A genome-wide survey was conducted to compare the number of NBS-LRR genes in flowering plant species with sequenced genomes. A phylogenetic tree of angiosperms was downloaded from the plaBi database (http://plabipd.de/portal/angiosperm-phylogenetic-view). The number of NBS-LRR genes identified and the total number of genes in each flowering plant species with a sequenced genome were manually retrieved and aligned with the phylogenetic tree.

3.2.2 IDENTIFICATION OF CONSERVED MOTIFS

The central NBS domain contains several conserved motifs such as P-loop, Kinase-1 and GLPL. Eight distinct motifs within the NBS domain have been reported in *A. thaliana* by subjecting the protein sequences of NBS domain to MEME (Multiple Expectation Maximization for Motif Elicitation; Bailey et al., 2006) (Meyers et al., 2003). A similar approach was used to identify homologous conserved motifs in NBS domains of chickpea. The protein sequence of the NBS domain from each NBS-LRR gene was retrieved and subjected to MEME for prediction of the conserved motifs in chickpea NBS domains.

3.2.3 GENE STRUCTURE, SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSES

The exon/intron structure of the chickpea NBS-LRR genes was retrieved from the general feature format (GFF) file of the chickpea genome annotation from NCBI. Multiple sequence alignments were conducted on the full length of the 121 NBS-LRR protein sequences using the default parameters of the ClustalW program. Due to a pairwise distance calculation problem, four protein sequences (*LOC101489470*, *LOC105851382*, *LOC101488657*, and *LOC101498409*) were removed from further analysis. A Neighbor-Joining (NJ) phylogenetic tree of 117 proteins was constructed with 1,000 bootstrap replications using MEGA7.0. Gene Structure Display Server (GSDS) was used to align the phylogenetic tree to the gene structure description of the NBS-LRR genes along with the domain positions. Similarly, to construct the phylogenetic tree using only the NBS domain, the NBS domain protein sequence file used for MEME analysis was used in the ClustalW program with the same parameters used for constructing the full length phylogenetic tree. The motifs identified in the MEME analysis were aligned to the NBS phylogenetic tree using iTOL software (http://itol.embl.de).

3.2.4 DISTRIBUTION AND CLUSTER ANALYSIS OF NBS-LRR GENES

The distribution of NBS-LRR genes was retrieved from their physical positions from the 'CDC Frontier' genome assembly v1. The genes were also mapped on the advanced version 'CDC Frontier' genome assembly v2 for comparison (Edwards, 2016). To define gene clusters, the following parameters were established: a cluster must contain at least two genes, the distance between two neighbouring NBS-LRR genes should be less than 200 kb and no more than eight genes should be positioned between neighbouring NBS-LRR genes.

3.2.5 CO-LOCALIZATION OF NBS-LRR GENES WITH QTLs FOR ASCOCHYTA BLIGHT RESISTANCE

The information of the chickpea ascochyta blight resistance QTLs were retrieved from the cool season legume database (https://www.coolseasonfoodlegume.org). The physical locations of the markers associated with ascochyta blight resistance QTLs in the chickpea genome were obtained via sequence similarity analysis of both forward and reverse primer sequences of each marker using NCBI BlastN. Only hits with 100% coverage of both query and subject were selected. Based on the physical position of the markers, the physical positions of the corresponding ascochyta blight resistance QTLs were inferred on both versions of the 'CDC Frontier' genome assembly. The physical positions of the two candidate genes *CaETR1* and *Ein3* tagged with QTL_{AR1} and QTL_{AR3} were also retrieved to confirm the physical location of their corresponding QTLs. The co-localization of the NBS-LRR genes

and ascochyta blight resistance QTLs were analyzed using Microsoft Excel. For visualization, a physical map of the chickpea genome was constructed by combining the distribution map of the NBS-LRR genes and the physical location of the ascochyta blight resistance QTLs using Mapchart v2.2 (Voorrips, 2002).

3.2.6 ASCOCHYTA BLIGHT SCREENING

The three chickpea genotypes 'CDC Corinne', 'CDC Luna' (both moderately resistant to ascochyta blight) and 'ICCV 96029' (susceptible) were used in the greenhouse trial to study the expression profile of selected NBS-LRR genes upon *A. rabiei* infection. The experiment was conducted in a completely randomized design. For the control and inoculated treatments, three biological replications (three separate plants) were used for each chickpea genotype and each time point. Three-week old seedlings were inoculated with monoconidial suspension of *A. rabiei* isolate AR-170. Approximately 3 mL of conidial suspension with a concentration of 2×10^5 conidia mL^{-1} was sprayed onto each plant using an air compressor. Control plants were mock-inoculated with water. Following inoculation, all plants were kept in a humidity chamber equipped with two humidifiers which maintained relative humidity of 100% for 48 h. Later all plants were moved to greenhouse benches equipped with an overhead misting system and all sides of the bench were covered with plastic sheets to maintain high humidity. Leaf samples from both inoculated and control plants were collected at 12, 24, 48, and 72 h post inoculation (hpi) from each of the three biological replicates of control and inoculated plants. Collected tissue samples were immediately frozen in liquid nitrogen and stored at -80°C prior to RNA extraction.

3.2.7 QUANTITATIVE REAL-TIME PCR (QRT-PCR)

Total RNA was extracted and treated with DNase I using SV Total RNA Isolation kit following the manufacturer's instruction (Promega, USA). Extracted RNA sample quantity was determined by an optical density reading at 260 nm and the OD260/OD280 absorption ratio using NanoDrop 800 UV-vis spectrophotometer (Thermo Fisher Scientific, Inc. USA). RNA integrity was checked with 1.5% agarose gel electrophoresis. Total RNA (1 μg) was reverse transcribed to cDNA using the SensiFAST cDNA synthesis kit (Bioline, Inc.). The cDNA used for qRT-PCR was diluted 5x with DNase/RNase free water. Specific primers were designed for each of the co-localized NBS-LRR genes in ascochyta blight resistance QTLs and five reference genes (*18SrRNA*, Elongation factor [*Ef1α*], *GAPDH*, Initiation factor [*IF4a*] and *ACTIN*) using IDT Primer quest tool (Integrated DNA Technologies, Inc.)

(Appendix 1). The primer pairs were designed to span exon-exon junctions with PCR product sizes between 55-180 bp, a primer sequence length of 18-25 nucleotides, T*m* between 50-60°C and a GC content of 50-60%. Each primer was tested on cDNA and genomic DNA samples to ensure amplification of the target region. Primer efficiencies of each target and reference gene were calculated based on tenfold serial dilutions of cDNA using the equation (1 + E) = 10 slope (Ramakers et al., 2003). The SensiFAST SYBR No-ROX kit was used for the target gene expression using an optical 384 well plate on the BIO-RAD CFX384 real-time PCR detection system (Bio-Rad laboratories) in accordance with the manufacturer's protocols. Two technical replications of each of the three independent biological replication per chickpea genotype at each time point were performed in a single plate along with controls (negative reverse transcription control [-RTC] and no template control [NTC]) for detection of DNA contamination or primer dimers). PCR product specificity of each gene was checked by melting curve analysis carried out by the PCR machine after 40 amplification cycles. All experimental samples for each amplicon had a single sharp peak at the amplicon melting temperature.

3.2.8 qRT-PCR DATA ANALYSIS

Among the tested five reference genes, *GAPDH* was selected and used to normalize the relative quantities of the target genes based on its consistency across different time points and genotypes. The comparative C_T method was used for the quantification of the expression of co-localized NBS-LRR genes in ascochyta blight resistance QTLs in which fold changes in expression were calculated by the $2^{-\Delta\Delta C_T}$ method (Schmittgen and Livak 2008). A mean fold change expression level of 2.0 was used as a cut-off point for up-regulation. Differentially expressed genes were clustered using hierarchical cluster analysis. The UPMG method was used to generate a dendrogram using *K*-means clustering with Cluster v3.0. The heatmap was constructed and viewed using Treeview v1.60. The complete procedure of the expression profiling is summarized in **Figure 3.1**.

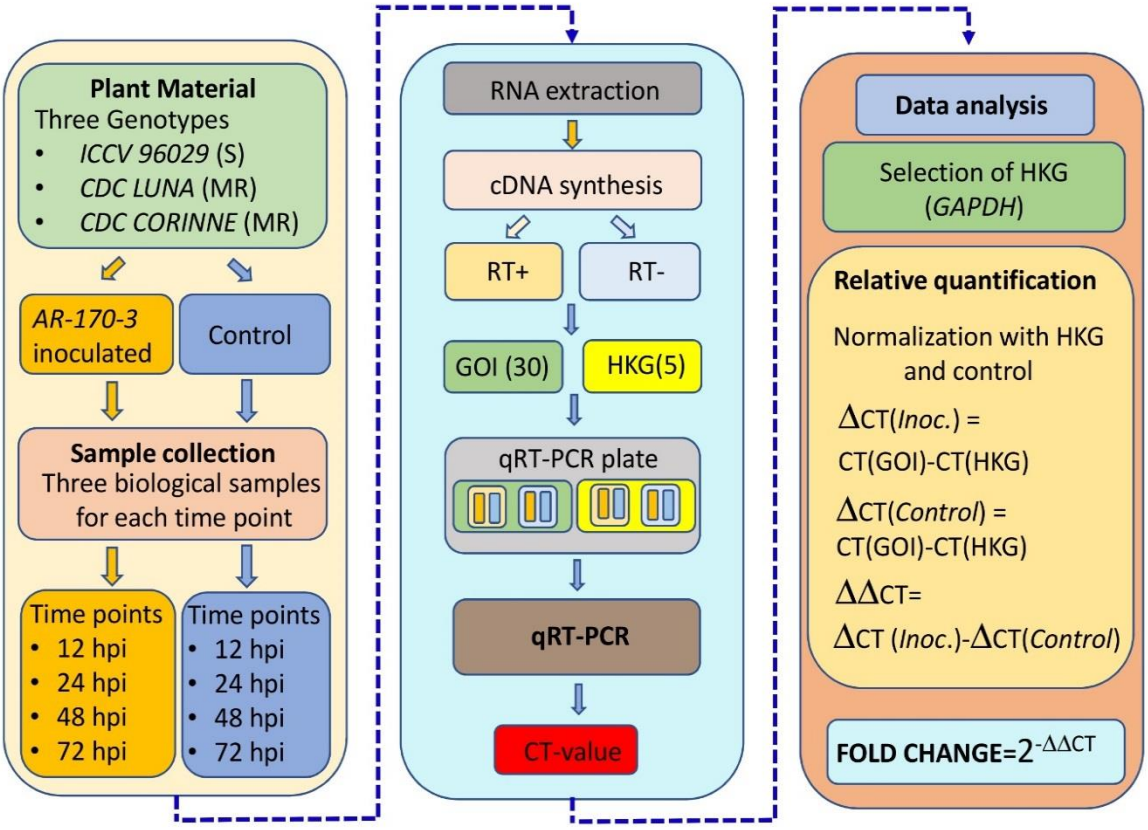


Figure 3.1. Overview of the experimental design and data analysis used for the expression profiling of the co-localized NBS-LRR genes within the known QTLs for ascochyta blight resistance.

3.3 RESULTS

3.3.1 IDENTIFICATION AND CLASSIFICATION OF CHICKPEA NBS-LRR GENES

A total of 121 NBS-LRR genes were identified in the 'CDC Frontier' genome assembly v1. In comparison to other plant species, the ratio of NBS-LRR genes to total genes is comparable to *A. thaliana*, *Lotus japonicus* and *Glycine max* (**Figure 3.2)**. Based on the protein domain combinations, the NBS-LRR genes were grouped into eight classes (**Table 3.1)**. Among the 121 genes, 98 were complete as they carried both the NBS and LRR domains while the remaining 23 genes were partial as they carried the NBS domain but lacked the LRR domain. The majority of the genes belong to the TNL class (39) followed by the CNL class (34) and the NL class (21). Other than TIR and CC domain in the N-terminal position, five genes with the RPW8 domain were identified and classified as RPW8-NBS-LRR (RNL [4]) and RPW8-NBS (RN[1]). Sixteen genes that only carried the central NBS domain and lacked both the N-terminal domain and the C-terminal LRRs were classified as NBS class.

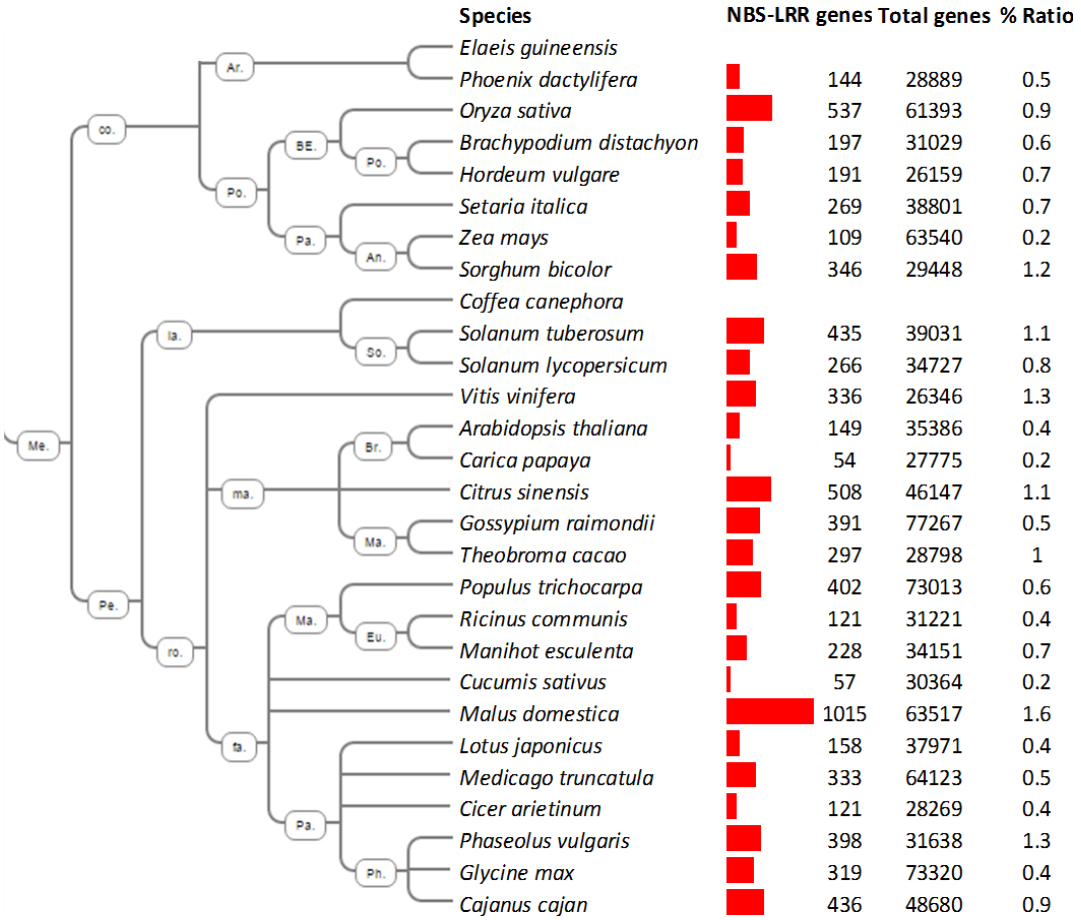


Figure 3.2. Genome-wide survey of the NBS-LRR genes in flowering plants. Phylogeny depicts the sequence similarity among diverse flowering species. A wide range of NBS-LRR gene frequency can be observed, spanning from 54 in papaya and 1015 in apple. Chickpea contains 121 NBS-LRR genes.

Table 3.1. Classification of the NBS-LRR genes in the chickpea genome.

Set	Class	No. of genes
With LRR	1. CC-NBS-LRR	34
	2. TIR-NBS-LRR	39
	3. RPW8-NBS-LRR	4
	4. NBS-LRR	21
Without LRR	5. CC-NBS	3
	6. TIR-NBS	3
	7. RPW8-NBS	1
	8. NBS	16
Total		121

Note: NBS: Nucleotide Binding Site, LRR: Leucine Rich Repeat, CC: Coiled-Coil, TIR: Toll/ Interleukin Receptor, RPW8: Resistance to Powdery Mildew 8

3.3.2 IDENTIFICATION OF CONSERVED MOTIFS WITHIN NBS DOMAIN

The MEME motif analysis within the chickpea NBS domain identified eight conserved motifs similar to the motif structure of the *A. thaliana* NBS domain. The eight major motifs varied in their divergence within and between the TNL and CNL classes (**Table 3.2**). Six conserved motifs (P-loop, Kinase-2, RNBS-B, RNBS-C, GLPL and MHDV) were consistently detected in each TNL and CNL class. Two motifs, RNBS-A and RNBS-D, were more diverse in their sequences, which distinguished the CNL and TNL class. All eight motifs in the NBS domain of each gene followed the strict motif order from P-loop to MHDV.

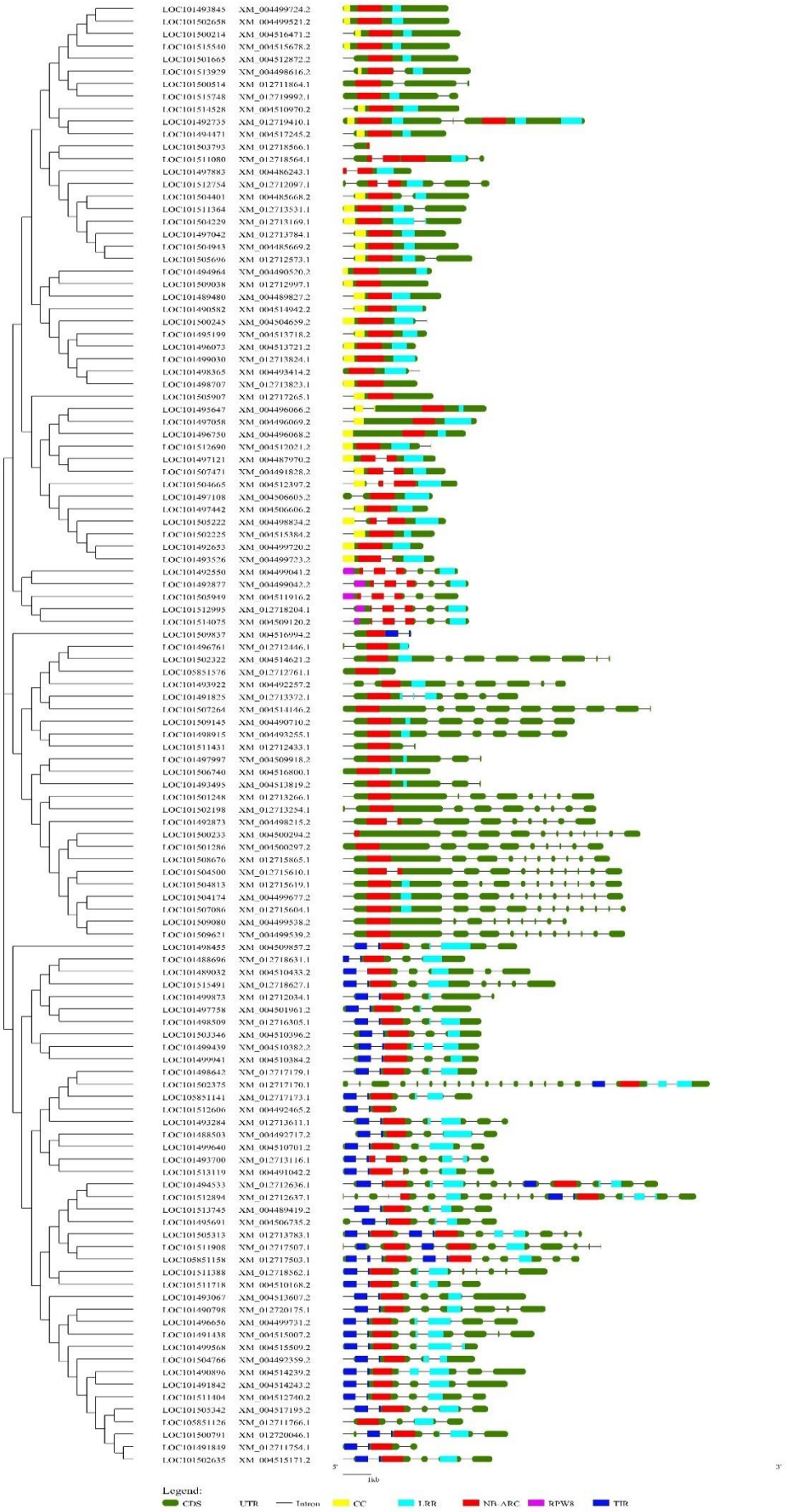
Table 3.2. Consensus sequence of the major motifs identified in the Chickpea NBS domain of the CNL and TNL proteins.

Motif	CNL	TNL
P-loop	VIPIVGMGGLGKTTLAQLVYND	LGIWGMGGIGKTTLAKAIYNKIXR
RNBS-A	DLKAWVCVSDDFDVLKVTKXI	FEGRCFLENVRENSE
Kinase-2	LQGKRFLLVLDDVWNEDY	IIKRRLCRKKVLLVLDDVDKLEQ
RNBS-B	PCGAKGSKILVTTRNQKVAS	WFGPGSRIIITTRDKHLLXGH
RNBS-C	HSLEXLSDEDCWSLFAKHAFR	YEVKELNEKESLELFSWHAFKQDX
GLPL	LEKIGKEIVKKCGGLPLAAVT	VVXYAGGLPLALEVLGSFLFGKDI
RNBS-D	DKKDLILLWMAEGFL	LDDTEKEIFLDIACF
MHDV	FVMHDLVHDLATLVSGEFYFR	MHDLLQDMGREIVREESPKEP

3.3.3 SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSIS

A NJ phylogenetic tree of 117 complete NBS-LRR proteins was constructed to examine their sequence and structural diversity among the NBS-LRR genes (**Figure 3.3**). The NJ tree displayed two clear clades which distinctly separated the TNL class from non-TNL class. The presence of TNL and non-TNL class in separate clades indicates the presence of structural and sequence variation among the genes belonging to these two classes. The TNL clade consists of three TNL sub-clades. The non-TNL clades were separated into CNL and NL sister clades. Further, the CNL clade was clearly separated into two sub-clades consisting of a CNL clade and a RPW8 clade. Phylogenetic clustering of genes with similar sequences from different chromosomes and the same chromosome was observed. The alignment of the phylogenetic tree to gene structure revealed that exon-intron structure tends to remain the same within the genes present in the same clade reflecting strong conservation of gene structure during evolution. The NJ tree constructed using only the NBS domain protein

sequences showed similar results to the complete protein NJ tree as the TNL clade was clearly separated from non-TNL clade (**Figure 3.4**). However, the RPW8 sub-clade was clustered with the NBS clades in the NJ tree of only NBS protein sequences, in contrast to the complete protein NJ tree in which the RPW8 sub-clade and the CC sub-clade were clustered together. Eight major motifs were identified in the NBS domain in the MEME analysis (**Table 3.2**), and the alignment of motif distribution with the phylogenetic tree showed that all genes follow strict motif order. Six conserved motifs were present in most of the NBS domain containing genes and two motifs (RNBS-A and RNBS-D) were more diverse in their sequence and distinguished the CNL and TNL class (**Table 3.2; Figure 3.4**).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5'
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Legend:
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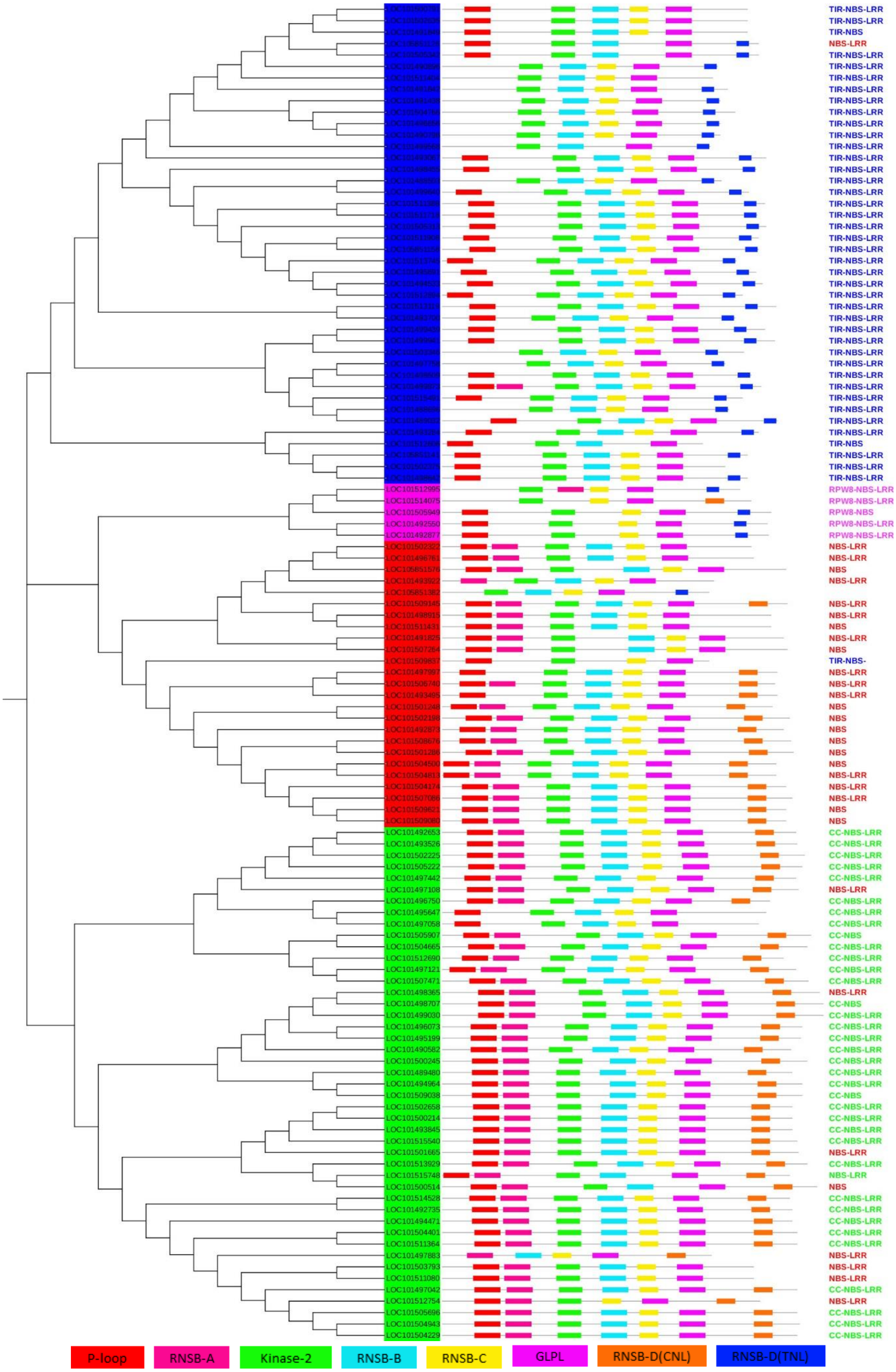
Intron
CC
LRR
NB-ARC
RPW8
TIR

Figure 3.3. A Neighbor-Joining phylogenetic tree depicting the sequence and structural diversity among the chickpea NBS-LRR genes aligned with the exon-intron structure of each gene along with the domain distribution. This tree was constructed using 117 complete NBS-LRR protein sequences with 1,000 bootstraps and evolutionary distance was calculated using the p-distance method. The gene structure was retrieved from the chickpea annotation and General feature file (GFF3) and the position of the domain in each gene was obtained from NCBI conserved domain database (CDD). The phylogenetic tree was aligned with gene structure along with domain position using GSDS. Different domains are indicated by different colors.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LOC101490582 CC-NBS-LRR
LOC101500245 CC-NBS-LRR
LOC101489480 CC-NBS-LRR
LOC101494964 CC-NBS-LRR
LOC101509038 CC-NBS
LOC101502658 CC-NBS-LRR
LOC101500214 CC-NBS-LRR
LOC101493845 CC-NBS-LRR
LOC101515540 CC-NBS-LRR
LOC101501665 NBS-LRR
LOC101513929 CC-NBS-LRR
LOC101515748 NBS-LRR
LOC101500514 NBS
LOC101514528 CC-NBS-LRR
LOC101492735 CC-NBS-LRR
LOC101494471 CC-NBS-LRR
LOC101504401 CC-NBS-LRR
LOC101511364 CC-NBS-LRR
LOC101497883 NBS-LRR
LOC101503793 NBS-LRR
LOC101511080 NBS-LRR
LOC101497042 CC-NBS-LRR
LOC101512754 NBS-LRR
LOC101505696 CC-NBS-LRR
LOC101504943 CC-NBS-LRR
LOC101504229 CC-NBS-LRR
P-loop
RNSB-A
Kinase-2
RNSB-B
RNSB-C
GLPL
RNSB-D(CNL)
RNSB-D(TNL)

Figure 3.4. The Neighbor-Joining phylogenetic tree constructed using only NBS domain sequences aligned with the distribution of conserved motifs identified in respective NBS domain and their respective gene class. Different motifs are indicated by different colors. Colour range for genes clades is based on the different N-terminal domain of gene classes as TIR (blue), RPW8 (pink), CC (green) and NBS (red).

3.3.4 DISTRIBUTION OF NBS-LRR GENES

The physical locations of the NBS-LRR genes were identified based on the chickpea gene annotation and GFF3 file. Using the 'CDC Frontier' genome assembly v1, 93 NBS-LRR genes were anchored on the eight chickpea chromosomes and 28 NBS-LRR genes were placed on the unanchored scaffolds. With the advanced genome assembly version v2, 109 NBS-LRR genes were physically mapped on the eight chickpea chromosomes and the remaining 12 NBS-LRR genes were located on the unplaced scaffolds. The chromosomal location of the NBS-LRR genes revealed an uneven distribution on the eight chickpea chromosomes and showed tandemly located gene clusters (**Figure 3.5**). Chromosome 5 has the highest number (29) of NBS-LRR genes (27% of mapped genes), while chromosome 8 has the lowest number (5) of NBS-LRR genes. At least one CNL gene was present on each chickpea chromosome while the TNL class was absent on chromosome 4. Out of the 121 NBS-LRR genes, 58 genes were present in 23 clusters each carrying two to four genes while 68 genes were present as singletons (**Table 3.3**). Among the 23 clusters, 18 were monophyletic clusters containing 45 genes and 5 were mixed clusters containing 13 genes. Out of 58 genes present in clusters, 32 were located on chromosome 5 (18) and 7(14). A maximum of four genes per cluster was found in each mono-cluster and mixed-cluster on chromosome 5 and 7, respectively.

Table 3.3. Cluster analysis of the NBS-LRR genes in chickpea.

Cluster Type	Cluster	Cluster Size (KB)	No. of Genes	Chr.	Gene ID
Mono-cluster	1	16.4	3	1	LOC101504943, LOC101504401, LOC101505696
	2	3.2	2	1	LOC101513119, LOC101493700
	3	0.8	2	2	LOC101501248, LOC101502198
	4	53.3	3	2	LOC101494533, LOC101512894, LOC101513745
	5	18.9	3	4	LOC101495647, LOC101496750, LOC101497058
	6	71.3	2	4	LOC101495199, LOC101496073
	7	2.9	2	4	LOC101492550, LOC101492877
	8	57.6	3	5	LOC101508676, LOC101500233, LOC101501286
	9	4.4	2	5	LOC101497758, LOC101498509
	10	21.9	2	5	LOC101509621, LOC101509080,
	11	144.1	4	5	LOC101507086, LOC101504174, LOC101504500, LOC101504813
	12	45.2	3	5	LOC101492653, LOC101493526, LOC101493845
	13	0.7	2	6	LOC105851141, LOC101502375
	14	40.3	2	6	LOC101511908, LOC105851158
	15	5.6	2	7	LOC101512995, LOC101514075
	16	11.9	3	7	LOC101488696, LOC101515491, LOC101489032
	17	199.2	3	7	LOC101499439, LOC101499941, LOC101503346
	18	20	2	7	LOC101490896, LOC101491842

Table 3.3. (Continued).

Cluster Type	Cluster	Cluster Size (KB)	No. of Genes	Chr.	Gene ID
Mixed-cluster	19	116	2	1	LOC101511364, LOC101512754
	20	12	3	3	LOC101498365, LOC101498707, LOC101499030
	21	18.5	2	5	LOC101497108, LOC101497442
	22	145.1	2	5	LOC101499568, LOC101500514
	23	47	4	7	LOC101503793, LOC101511080, LOC101511388, LOC101511718
Total clusters	23		58		
Non-clustered		63			
Total genes			121		

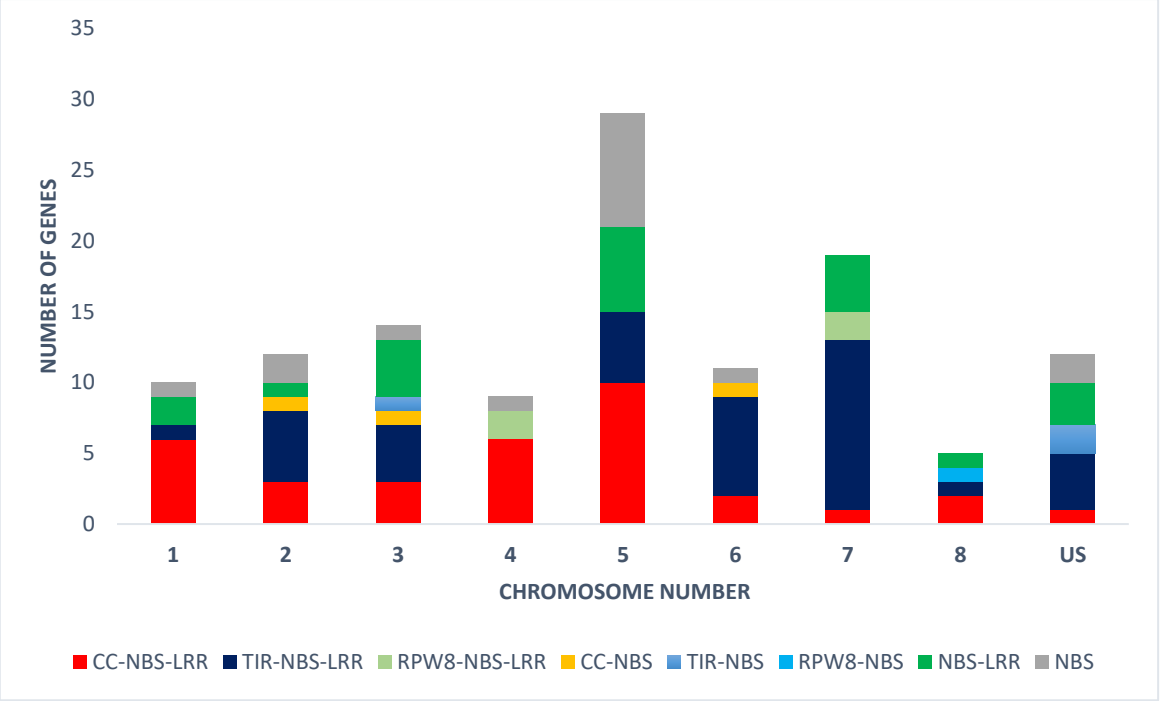

Figure 3.5. Distribution of the NBS-LRR genes on each chickpea chromosome (1-8) and unplaced scaffold (US). Each gene class was denoted by different color.

3.3.5 CO-LOCALIZATION OF NBS-LRR GENES WITH ASCOCHYTA BLIGHT QTLs

Based on the physical position of the SSR markers on the chickpea chromosomes, a total of 16 QTLs previously reported for ascochyta blight resistance were mapped on chromosomes 2, 3, 4, 5, 6 and 8 (**Table 3.4**). Nine ascochyta blight resistance QTLs were co-localized with NBS-LRR genes. Out of the nine QTLs, three QTLs (Cho et al., 2004; [*QTL-AR2*] Iruela et al., 2006; [*AB-Q-SR-4-2*] Sabbavarapu et al., 2013) were mapped on chromosome 4, three QTLs ([*AB-Q-APR-6-1*, *AB-Q-APR-6-2*] Sabbavarapu et al., 2013; [*QTL4*] Tar'an et al., 2007) were mapped on chromosome 6 and one QTL each on chromosome 2 ([QTL1] Anbessa et al., 2009), chromosome 3 ([*QTL2*] Tar'an et al., 2007) and chromosome 8 ([*QTL5*] Anbessa et al., 2009). In total, 30 NBS-LRR genes were co-located between the flanking markers of these nine ascochyta blight resistance QTLs (**Figure 3.6**). Among the co-localized NBS-LRR genes, 24 genes were complete genes i.e. these genes carry all essential domains for their independent functions. Among these 24 genes, 13 belong to TNL class, eight belong to the CNL class and three belong to the NL class. The remaining six co-localized genes which belonged to the RN class (1), CN class (2) and NBS class (3) were incomplete lacking the LRR domain. The majority of the genes (17) co-localized with ascochyta blight resistance QTLs were present in clusters of 2 to 3 genes. On chromosome 2,

QTL1 (Anbessa et al., 2009) co-localized with the three mono-clusters 2, 3 and 4, consisting of two TNL, two NBS and three TNL class genes, respectively (**Table 3.3**). The *QTL2* (Tar'an et al., 2007) on chromosome 3 overlaps with the mixed-cluster 20 consisting of three genes, each from the NL, CN and CNL class. On chromosome 4, the QTL reported by Cho et al., (2004) co-localized with cluster 5 consisting of three CNL class genes. Two mono-clusters, cluster 13 and cluster 14 each consisting of two TNL class genes were co-localized with *AB-Q-APR-6-2* (Sabbavarapu et al., 2013) on chromosome 6.

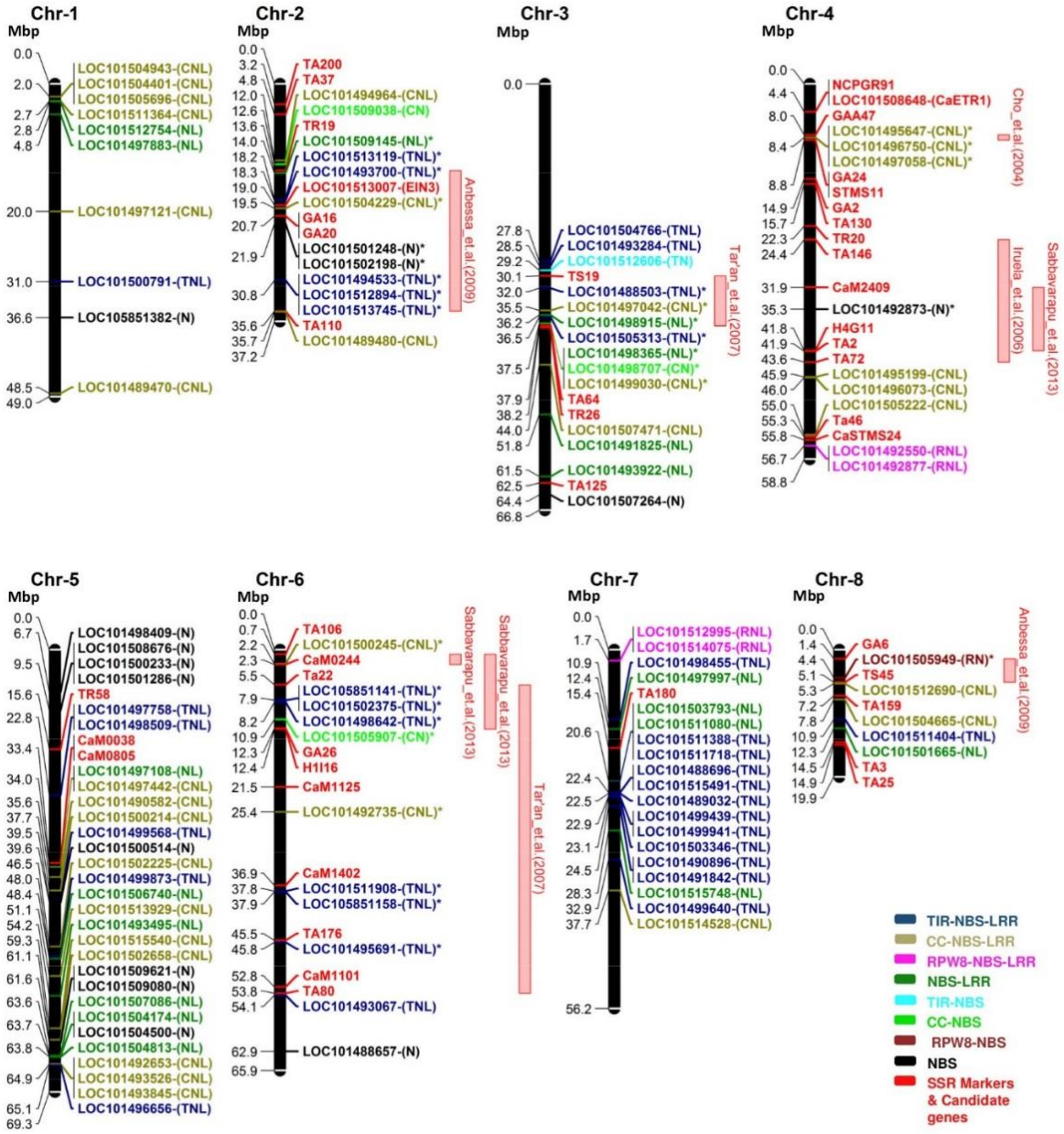

Figure 3.6. The distribution of NBS-LRR genes on the physical map of 'CDC Frontier' v2 along with the position of the markers corresponding to the physical positions of quantitative trait loci (QTLs) for ascochyta blight resistance. Eight chromosomes (Chr) of chickpea are represented as black bars. Gene and marker names are shown on the right side and their physical positions in megabase pair (Mbp) are shown on the left. QTLs with co-localized NBS-LRR genes are shown as red bars on the right side of the chromosomes. Thrity co-localized NBS-LRR genes are indicated with (*) along with their name.

Table 3.4. List of previously reported quantitative trait loci (QTLs) associated with resistance to ascochyta blight and their location on the chickpea physical map of 'CDC Frontier' v2.

Reference	QTL name	Population	Linkage Group	Closest Marker	Chromosome	Start (bp)	End (bp)
Cho et.al.,2004	-	P1359075 x FLIP84-92C	LG 2	TA200	Ca2	3181630	3181404
				TA37	Ca2	4794769	4794514
Cho et.al.,2004	-	P1359075 x FLIP84-92C	LG 2	GA16	Ca2	20725707	20725486
				GA20	Ca2	20725716	20725568
Anbessa et.al.,2009	QTL1	ICCV96029 x CDC Luna	LG 2	TR13/TR19	Ca2	13605945	13606125
				TA110	Ca2	35645481	35645662
Tar'an et.al.,2007	QTL2	ICCV96029 x CDC Frontier	LG 3	TS19	Ca3	30120220	30120309
				TA64	Ca3	37876189	37876000
Anbessa et.al.,2009		ICCV96029 x AMIT	LG 3	TA64	Ca3	37876189	37876000
				TR26	Ca3	38224538	38224343
Udupa et.al., 2003	-	ILC1272 x ILC3279	LG 4	TA130	Ca4	15658926	15658717
				TR20	Ca4	22340178	22340026
Cho et.al.,2004	-	P1359075 x FLIP84-92C	LG 4	GA24	Ca4	8802287	8802468
				GAA47	Ca4	8006877	8007024
Sabbavarapu et.al., 2013	AB-Q-SR-4-2	C214 x ILC3279-F2	LG 4	CaM2049	Ca4	31877378	31573954
				H4G11	Ca4	41777447	41777620
Iruela et.al.,2006	QTL-AR2	ILC3279 x WR315	LG 4	TA146	Ca4	24367557	24367586
				TA72	Ca4	43563684	43563874
Madrid et.al., 2012	QTL_AR1	WR315 x ILC3279	LG 4	NCPGR91	Ca4	4411683	4411370
				GAA47	Ca4	8006877	8007024
Sabbavarapu et.al., 2013	AB-Q-APR-5B	C214 x ILC3279	LG 4	CaSTMS11	Ca4	8802599	8802388
				TA130	Ca4	15658926	15658717
Sabbavarapu et.al., 2013	AB-Q-APR-5B	C214 x ILC3279	LG 5	CaM0038	Ca5	33381283	33381116
				CaM0805	Ca5	33387179	33386904

Table 3.4. (Continued).

Reference	QTL name	Population	Linkage Group	Closest Marker	Chromosome	Start (bp)	End (bp)
Sabbavarapu et.al., 2013	AB-Q-APR-6-1	C214 x ILC3279	LG 6	TA106	Ca6	685713	685506
				H1I16	Ca6	12381876	12381715
Sabbavarapu et.al., 2013	AB-Q-APR-6-2	C214 x ILC3279	LG 6	TA106	Ca6	685713	685506
				CaM0244	Ca6	2274651	2274456
Tar'an et.al.,2007	QTL4	ICCV96029 x CDC Frontier	LG 6	TA22	Ca6	5494172	54941700
				TA80	Ca6	53832720	53832897
Anbessa et.al.,2009	QTL5	ICCV96029 x CDC Corinne	LG 8	GA6	Ca8	1428366	1428214
				TS45	Ca8	5096421	5196641

3.3.6 EXPRESSION PROFILING OF NBS-LRR GENES

Among the 30 co-localized NBS-LRR genes in nine ascochyta blight resistance QTLs, 27 genes showed differential expression at least at one-time point after inoculation compared to the water-inoculated control in each of the three genotypes 'CDC Luna', 'CDC Corinne', and 'ICCV 96029' (**Figure 3.7**). The expression of the remaining three genes (*LOC101493700*, *LOC101513119*, and *LOC101494533*) was below the cut-off level at all time points in all genotypes. In 'ICCV 96029', the highest number of genes (17) were up-regulated at 12 hpi, while 7 NBS-LRR genes were up-regulated in 'CDC Luna' and only 2 in 'CDC Corinne' at that time point (**Figure 3.8**). In 'CDC Luna', the highest number of genes (17) was up-regulated at 24 hpi, while 11 and 12 genes were up-regulated in 'ICCV 96029' and 'CDC Corinne', respectively (**Figure 3.8**). In 'CDC Corinne', the highest number of NBS-LRR genes (18) was up-regulated at 48 hpi, while 10 and 14 genes were up-regulated in 'ICCV 96029' and 'CDC Corinne', respectively (**Figure 3.8**). At 72 hpi 14, 3 and 20 genes showed up-regulation in 'ICCV 96029', 'CDC Luna', and 'CDC Corinne', respectively. On average, most genes showed up-regulation at 12 hpi and 24 hpi in 'ICCV 96029', at 24 hpi and 48 hpi in 'CDC Luna' and at 48 hpi and 72 hpi in 'CDC Corinne'. Five genes showed genotype-specific expression. Two of those (*LOC101509145* [**Figure 3.9 (a)**], *LOC101498915*) showed up-regulation only in 'CDC Corinne' and were down-regulated or did not change when compared to the control sample in 'ICCV 96029' and 'CDC Luna'. In contrast, the other three genes (*LOC101513745* [**Figure 3.9 (b)**], *LOC101512894*, and *LOC101497042*) showed up-regulation in 'ICCV 96029' and 'CDC Luna', but no change or down-regulation was observed in 'CDC Corinne'. One gene (*LOC101505949*) was constantly expressed in all three genotypes and at all time points, except at 12hpi in 'CDC Corinne'. In terms of the levels of expression, a range of 2-13 fold changes in expression was observed. The highest fold change in expression was observed for *LOC101498365* and *LOC101511908* in 'CDC Corinne' at 72 hpi when compared to the mock-inoculated control, followed by a 12-fold change in *LOC101505907* and *LOC101511908* in 'CDC Luna' and *LOC101500245* in 'ICCV 96029'. Expression profiling of the NBS-LRR genes allowed to differentiate the three chickpea genotypes. The susceptible cultivar 'ICCV96029' was separated from the moderately resistant cultivars 'CDC Corinne' and 'CDC Luna'. The moderately resistant cultivars were distinguished from each other with respect to the up-regulation of the NBS-LRR genes at different time points after inoculation **(Figure 3.9 [c, d])**.

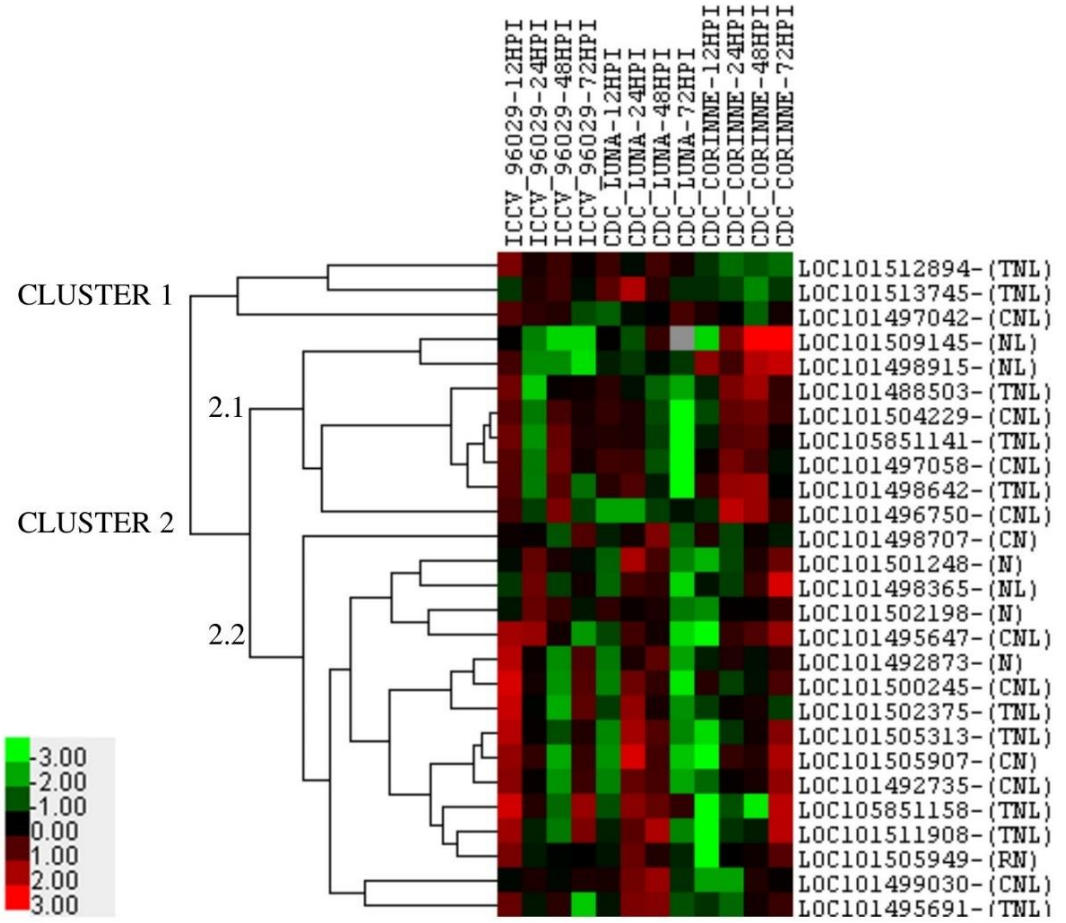

Figure 3.7. Heatmap of 27 NBS-LRR genes representing the mean fold change expression profiles at four different time points in chickpea cultivars 'ICCV 96029', 'CDC Luna' and 'CDC Corinne' after infection with *A. rabiei* isolate AR-170. The mean fold change expression values were calculated after normalization with the reference gene (GAPDH) and non-infected control samples. Red represents up-regulation, black represents no change and green represents down-regulation as presented in color bar.

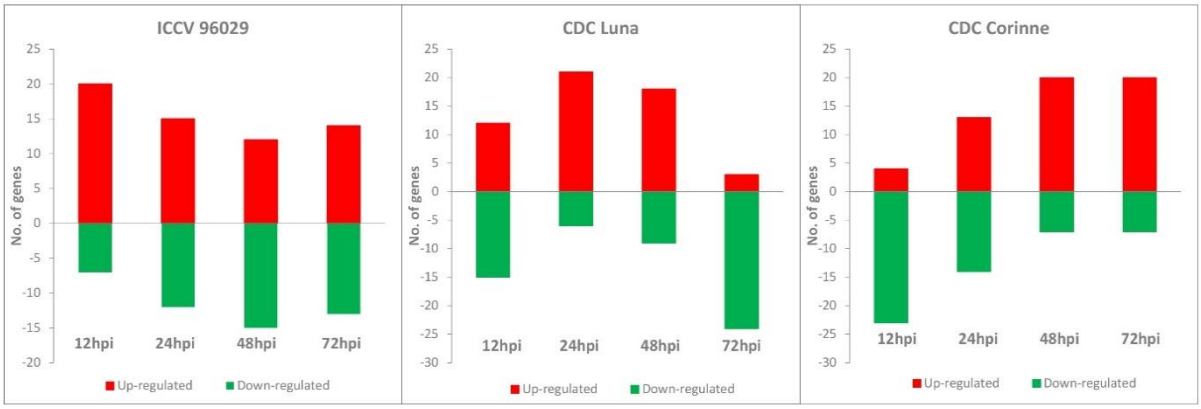

Figure 3.8. The total number of up-regulated genes (red) and down-regulated genes (green) at four different time points in three chickpea cultivars 'ICCV 96029', 'CDC Luna' and 'CDC Corinne' after infection with *A. rabiei* isolate AR-170.

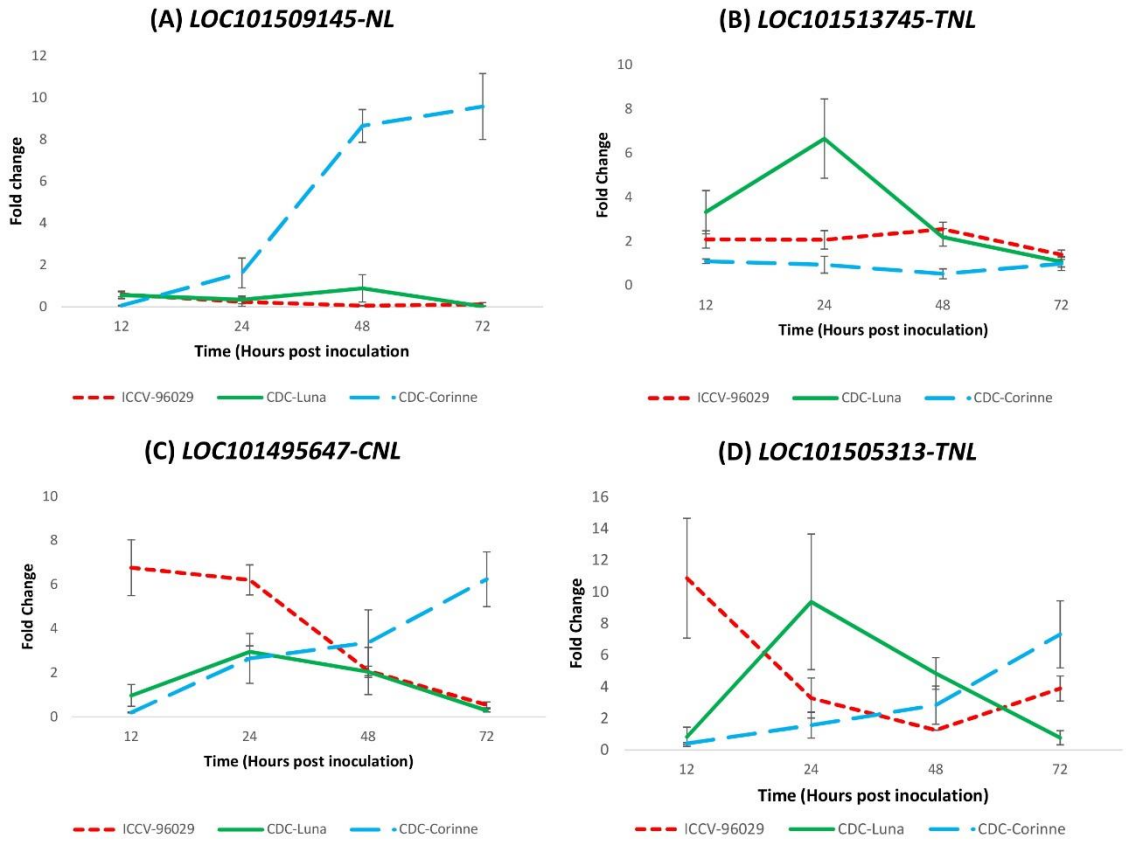

Figure 3.9. The mean fold change expression profiles of four NBS-LRR genes showing genotype-specific expression (a and b) and time point specific expression pattern (c and d) in chickpea cultivars 'ICCV 96029', 'CDC Luna' and 'CDC Corinne' after infection with *A. rabiei* isolate AR-170 at four different time points.

3.3.7 PATTERN OF GENE EXPRESSION WITHIN AND AMONG GENOTYPES

Cluster analysis of the 27 NBS-LRR genes revealed the underlying gene expression patterns of genotypes at different time points (**Figure 3.7**). Two major clusters were observed, cluster 1 with 3 NBS-LRR genes and cluster 2 with 24 NBS-LRR genes. A common pattern of gene expression was observed among the three genes *LOC101512894*, *LOC101513745*, and *LOC101497042* in cluster 1. These genes were only up-regulated in genotypes 'ICCV 96029' and 'CDC Luna', but their expression was below the cut-off limit in 'CDC Corinne'. Cluster 2 consisted of the two sub-clusters 2.1 and 2.2. Eight genes were present in cluster 2.1. Among these, *LOC101509145* and *LOC101498915* showed contrasting expression patterns compared to genes present in cluster1 as they only showed up-regulation in 'CDC Corinne', which suggests that these genes are specific to 'CDC Corinne' in response to ascochyta blight infection. The other six genes in cluster 2.1 showed a common pattern of up-regulation at 12 and 48 hpi in 'ICCV 96029', while in 'CDC Luna' these genes showed up-regulation at 12

and 24 hpi and in ‘CDC Corinne’ at 24 and 48hpi. The sub-cluster 2.2 consisted of 16 genes, and with few exceptions, most genes were up-regulated at 12, 24 and 72 hpi in ‘ICCV 96029’, 24 and 48 hpi in ‘CDC Luna’, and at 48 and 72 hpi in ‘CDC Corinne’.

3.4 DISCUSSION

Ascochyta blight is one of the major yield limiting factors of chickpea production worldwide. However, disease severity is more significant in areas with cooler and wet growing seasons such as Western Canada (Tar’an et al., 2007). Limited success has been achieved in developing ascochyta blight resistant cultivars due to lack of complete resistance in chickpea germplasm. To date, several QTLs associated with ascochyta blight resistance have been identified in diverse genetic backgrounds. Yet, the precise mechanism of resistance to ascochyta blight is still unknown.

Numerous plant disease resistance genes including NBS-LRR genes that play a major role in resistance against a diverse array of pathogens have been identified and cloned in many plant species (Hammond-Kosack and Jones 1997). The majority of the NBS-LRR genes are known to provide resistance against biotrophic pathogens following a “gene-for-gene” or “guard” model of host-pathogen interaction leading to the activation of salicylic acid (SA) pathway and its defense responses (Glazebrook, 2005). Knowledge of resistance mechanisms against necrotrophic pathogens was initially limited to phytotoxin production and activation of the jasmonic acid (JA) and ethylene pathways (Glazebrook, 2005). An association of NBS-LRR genes with susceptibility against necrotrophic pathogen has been observed in different studies (Lorang et al. 2007; Nagy and Bennetzen 2008; Faris et al. 2010). Recent studies also showed the involvement of NBS-LRR genes in resistance reactions against necrotrophic pathogens. For example, the *A. thaliana* gene *RLM3* of the TNL class provides resistance against the three necrotrophic fungi *Botrytis cinerea, Alternaria brassicicola,* and *Alternaria brassicas,* and the hemibiotrophic fungus *Leptosephaeria maculans* (Staal et al., 2008). In wheat, over-expression of *TaRCR1,* a member of the CNL class, increased resistance against the necrotrophic fungus *Rhizoctonia cerealis* (Zhu et al., 2016).

The involvement of NBS-LRR genes against ascochyta blight infection in chickpea has not been reported so far. The chickpea genome consists of 121 NBS-LRR genes, which is about 0.43% of the total 28,269 annotated genes. The frequency of NBS-LRR genes is highly variable among plant species and can be as low as 0.21% in *Carica papaya* (Porter et al.,

2009) and as high as 1.6% in *Malus domestica* (Arya et al., 2014). The chickpea NBS-LRR gene frequency falls within this range. Several studies showed that there is no correlation between the NBS-LRR gene frequency and the genome size or the total number of annotated genes. One hypothesis for the presence of a low number of NBS-LRR genes is a potential fitness cost or lethal effects of NBS-LRR genes on plant cells which restrict the number of NBS-LRR genes in the plant genome (Zhang et al., 2016). Despite the relatively low number of NBS-LRR genes, most of the genes in the chickpea NBS-LRR gene family possess the essential conserved domains observed in other plant species. Out of the 121 NBS-LRR genes, 98 genes encode proteins consisting of both NBS and LRR domains and the remaining 23 genes were incomplete. The presence of all necessary structural motifs indicates the potential for their functioning. However, truncated or incomplete genes have also been reported to have a function in co-operation with complete genes. For example, the two tandem NBS-LRR genes *RPP2A* and *RPP2B* are required to provide resistance against *Peronospora parasitica* isolate Cala2 in *A. thaliana* (Sinapidou et al., 2004). *RPP2A* is an incomplete TIR-NBS gene with a truncated LRR domain whereas *RPP2B* is a complete gene. Both genes complement each other by providing recognition specificity or signaling lacking by its partner and confer resistance against isolate Cala2. Up-regulation of truncated NBS-LRR genes upon ascochyta blight infection was also observed in this study.

The chickpea NBS-LRR gene family was grouped into eight major classes based on their domain architecture (Meyers et al., 2003). In general, the TNL class is often lacking in monocot species (Shao et al., 2016). Chickpea being a dicot species contains both TNL and CNL class genes and the numbers of TNLs (39) were higher than those of the CNLs (34), a pattern similar to *A. thaliana, Medicago truncatula, Glycine max,* and other dicot species. This indicates that through evolution NBS-LRR genes diversified significantly between monocots and dicots. The evolutionary divergence of the TNL from non-TNL/CNL class has been observed in many studies (Ameline-Torregrosa et al., 2008; Lozano et al., 2015; Meyers et al., 2003). It was also observed that genes with a RPW8 domain formed a separate sub-clade within the CNL clade which supports the functional divergence of the RPW8 from common CNL genes (Collier et al., 2011). Phylogenetic analysis indicated that chickpea NBS-LRR genes followed a similar pattern. The analysis showed that different clades represent the structural differences among these classes, for example among the non-TNL clades, the NL clade was separated from the CNL clade. The phylogenetic analysis supports

the criteria of gene classification into TNL, CNL, RNL and NL and similar classes that lack the C-terminal LRR domain.

The NBS-LRR genes were unevenly distributed across all chickpea chromosomes. For example, chromosome 5 contains the highest number of NBS-LRR genes and chromosome 8 has the lowest. It has been frequently observed that NBS-LRR genes are present in clusters, which may contribute to their genetic variation and rapid evolution (Hulbert et al., 2001). In chickpea, nearly half of the NBS-LRR genes (48%) were present in clusters. Among these clustered genes, mono-clusters (78%) were more abundant than mixed clusters which may be a reflection of their evolution through tandem duplications. Majority (55%) of the gene clusters were found on chromosome 5 and 7. Another significance of clustering of NBS-LRR genes is that tandem clustering of functionally related genes facilitates co-expression to form functional heterodimers which may interact with pathogen effector molecules to initiate resistance as observed in *Oryza sativa* (Ashikawa et al., 2008) and *A. thaliana* (Sinapidou et al., 2004).

Co-localized genes in QTL regions have been successfully used to identify candidate genes associated with different traits. In chickpea, *CaETR1* and *Ein3* were identified as candidate genes for ascochyta blight resistance based on their co-localization with QTL_{AR1} and QTL_{AR3}, respectively (Madrid et al., 2012, 2014). In soybean, the strong positive correlation between the number of NBS-LRR genes and the disease resistance QTLs on each chromosome reflects the contribution of this gene family in soybean disease resistance (Kang et al., 2012). This study tested the potential involvement of NBS-LRR genes in ascochyta blight resistance based on QTL co-localization and expression analysis. Thirty NBS-LRR genes were found to be co-localized with the physical position of nine ascochyta blight resistance QTLs on chromosome 2, 3, 4, 6, and 8. Clusters of the NBS-LRR genes were identified within the ascochyta blight QTLs. For example, on chromosome 4, a cluster of three CNL class genes was co-localized with an ascochyta blight resistance QTL (Cho et al., 2004). All three CNL genes showed high sequence similarity with RPP13 in *A. thaliana* which provides resistance to powdery mildew (Bittner-Eddy and Beynon, 2001). Previously it was reported that tandem clustering of NBS-LRR genes facilitates co-expression and provide effective resistance in *Oryza sativa* and *A. thaliana* against *Magnaporthe grisea* and *Peronospora parastica*, respectively (Ashikawa et al., 2008; Sinapidou et al., 2004). Co-expression of the NBS-LRR genes present in the cluster was also observed in this study. *LOC101501248* and *LOC101502198* of cluster 3 present in QTL1 on chromosome 2 showed similar induction

patterns in each chickpea genotype. *LOC101498365*, *LOC101498707*, and *LOC101499030* of cluster 2 co-located with QTL2 on chromosome 3 showed a similar expression pattern in each genotype. Co-expression of these clustered genes reflects their potential involvement in common resistance mechanism.

The *A. rabiei* isolate AR-170 infects both the susceptible and the moderately resistant genotypes as evident by the germination and constant growth of the fungus, and production of necrotic lesions in all three genotypes (**Appendix 2**). The moderately resistant genotypes 'CDC Luna' and 'CDC Corinne' showed delayed symptom development in comparison to the susceptible genotype 'ICCV 96029'. The majority of the co-localized NBS-LRR genes in ascochyta blight resistance QTLs showed differential expression in at least one genotype at one time point compared to the control. However, up-regulation of these genes were observed early during the infection process in the susceptible genotype compared to the resistant genotypes, which correlates with the disease progression on these genotypes. Genotype-specific expression patterns of some of the NBS-LRR genes was also observed. Two NBS-LRR genes (*LOC101509145* and *LOC101498915*) were up-regulated only in the moderately resistant cultivar 'CDC Corinne'. One gene (*LOC101505949*) co-localized with *QTL5* (Anbessa et al., 2009) on chromosome 8 showed up-regulation in all *A. rabiei* inoculated samples, except at 12 hpi in 'CDC Corinne'. This gene had very high sequence similarity with the *A. thaliana ADR1*-like genes (*AT4G33300*). The *A. thaliana ADR1* gene encodes an NBS-LRR which belongs to a special lineage of CNLs, a RPW8-NBS-LRR (RNLs). Characterization of a *A. thaliana* mutant, designated as *Activated Disease Resistance* (*ADR1),* showed elevated levels of SA and reactive oxygen intermediates (ROI), and provided broad resistance against the biotrophic pathogens *P. parasitica* and *E. cichoracea* (Grant et al., 2003). The presence of homolog of *ADR1* which provide resistance to biotrophic pathogens in ascochyta blight resistance QTL suggests common defense mechanism might be involved in providing resistance against biotrophic and necrotrophic pathogens.

In addition to NBS-LRR genes, other genes invovled in disease resistance may also be present within the ascochyta blight resistance QTL interval, such as *Ein3* that co-localizes with the NBS-LRR genes in *QTL2* and QTL_{AR3} (Anbessa et al., 2009; Madrid et al., 2014). *Ein3* is a plant-specific transcription factor which plays an important role in mediating ethylene responses (Madrid et al., 2014). As several signaling molecules including ethylene, SA, and JA are involved downstream of the NBS-LRR proteins (McHale et al., 2006), it is

likely that the ethylene pathway and the NBS-LRR genes are involved in providing resistance to ascochyta blight. Therefore, it would be interesting to further explore the interaction between the NBS-LRR and the ethylene pathway.

In summary, 121 NBS-LRR genes were identified and classified into eight distinct classes in the chickpea genome. The research demonstrated the potential involvement of NBS-LRR genes in response to *A. rabiei* based on their co-localization with known QTLs for ascochyta blight resistance and based on their expression profiles. This study provides resources for further functional analyses to validate the association of NBS-LRR genes with disease resistance in chickpea.

CHAPTER 4

GENETIC MAPPING OF QUANTITATIVE TRAIT LOCI (QTLs) AND NBS-LRR GENES ANALYSIS FOR ASCOCHYTA BLIGHT RESISTANCE USING THREE RECOMBINANT INBRED LINE (RIL) POPULATIONS

CHAPTER 4: GENETIC MAPPING OF NBS-LRR GENES AND QUANTITATIVE TRAIT LOCI (QTLs) ANALYSIS FOR ASCOCHYTA BLIGHT RESISTANCE USING THREE RECOMBINANT INBRED LINE (RIL) POPULATIONS.

4.1 INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a self-pollinated, annual diploid (2n=16) species with a genome size of 738 Mb (Arumuganathan and Earle, 1991). Chickpea is the world's second most important food legume. It is an excellent and inexpensive source of protein, dietary fibers, complex carbohydrates, vitamins, folate and minerals and its consumption has several potential health benefits (Jukanti et al., 2012; Roy et al., 2010). The chickpea crop can fix nitrogen in symbiosis with rhizobium and improves soil fertility. On a global scale, more than 90% of chickpea production occurs in the semi-arid tropics of Asia and Africa and the remaining production comes from Australia, Europe, North and South America (FAOSTAT, 2014). As chickpea requires minimal input of irrigation, fertilizers and pesticides, it is mainly grown by resource-poor farmers on marginal lands in developing Afro-Asian countries. Global demand and production of chickpea have increased due to the crop's nutritional benefits, improvement in soil health, crop diversification and premium prices in the international markets, especially for large seeded kabuli chickpea (Gaur et al., 2012). Although the average global chickpea yield has increased from 683 kg ha^{-1} to 966 kg ha^{-1} in the past decades (1990-2014), it is still below the yield potential of the crop (FAOSTAT, 2014). Breeding efforts have resulted in over 350 chickpea cultivars with improved yield (Gaur et al., 2012), but unstable production due to susceptibility to several biotic (ascochyta blight, fusarium wilt, pod borer) and abiotic stresses (drought, salinity and cold) is still a major concern for the adoption of this crop by farmers (Millan et al., 2006).

Ascochyta blight is one of the most important and widespread diseases that impacts chickpea yields and degrades crop quality. This disease is caused by the ascomycete fungal pathogen *Ascochyta rabiei* (Pass.) Labr., which infects all above ground plant parts at any plant growth stage (Pande et al., 2005). The destructive potential of *A. rabiei* is most severe under favorable weather conditions (cool and humid) at the flowering stage which can result in near complete yield loss (Sharma and Ghosh, 2016). Ascochyta blight epidemics resulted in a decline in the area under chickpea cultivation in Canada (Chandirasekaran et al., 2009), the USA (Kaiser et al., 1994), Australia (Bretag et al., 2008) and Latin America (Kaiser et al., 2000). Among the different disease management options, breeding for resistance to ascochyta

blight is considered the most cost-efficient method of control (Rubiales and Fondevilla, 2012).

Breeding for ascochyta blight resistance has been a major challenge for chickpea breeders. A major factor slowing down the breeding efforts is the presence of the sexual stage in the life cycle of *A. rabiei*. This contributes to high genetic variability in pathogen populations due to sexual recombination which results in new pathotypes. Another impediment to fast progress is the lack of complete resistance to ascochyta blight in cultivated and wild chickpea germplasm, so most chickpea breeding programs rely on the partially resistant sources which show low degree of infection (Sharma and Ghosh, 2016). A third factor is the complex genetic basis of ascochyta blight resistance. Numerous studies reported the involvement of varying numbers of genes controlling ascochyta blight resistance in chickpea in different cultivars, based on inoculation with different fungal isolates and methods of disease screening (Sharma and Ghosh, 2016). The initial studies indicated that the inheritance of genetic resistance to ascochyta blight is qualitative and governed by a single dominant gene in desi chickpea (Vir et al., 1975), whereas in kabuli chickpea it was either controlled by a single recessive gene (Singh and Reddy, 1989, 1990) or a dominant gene (Singh and Reddy, 1983; Tewari and Pandey, 1986). Other studies reported that the inheritance of ascochyta blight resistance was governed by two dominant complementary genes (Ahmad et al., 1952), two complementary dominant genes and one recessive and one dominant independent gene (Dey and Singh, 1993), and three major genes with complementary effects and other minor genes (Tekeoglu et al., 2000), depending on the source of resistance.

Later studies suggested that several QTLs are involved in governing resistance to ascochyta blight. To date, several QTLs have been identified on LG 1 (Daba et al., 2016; Flandez-Galvez et al., 2003; Santra et al., 2000), LG 2 (Anbessa et al., 2009; Cho et al., 2004; Cobos et al., 2006; Daba et al., 2016; Flandez-Galvez et al., 2003; Udupa and Baum, 2003), LG 3 (Anbessa et al., 2009; Aryamanesh et al., 2010; Daba et al., 2016; Flandez-Galvez et al., 2003; Tar'an et al., 2007), LG 4 (Aryamanesh et al., 2010; Cho et al., 2004; Daba et al., 2016; Iruela et al., 2006; Lichtenzveig et al., 2006; Madrid et al., 2014; Sabbavarapu et al., 2013; Stephens et al., 2014; Tar'an et al., 2007; Udupa and Baum, 2003), LG 5 (Sabbavarapu et al., 2013), LG 6 (Daba et al., 2016; Sabbavarapu et al., 2013; Santra et al., 2000; Tar'an et al., 2007), LG 7 (Daba et al., 2016) and LG 8 (Anbessa et al., 2009; Daba et al., 2016; Lichtenzveig et al., 2006). Since QTLs for ascochyta blight were identified on all eight

chickpea chromosomes, genes controlling ascochyta blight resistance are distributed across the genome.

Despite the abundant information on QTLs, knowledge of the genes responsible for ascochyta blight resistance is still limited. The availability of the draft genome sequence of desi (Jain et al., 2013), kabuli (Varshney et al., 2013c) and wild chickpea (*Cicer reticulatum* L.) (Gupta et al., 2016) has paved the path to anchor genetic maps and position QTLs onto the physical map. Using this approach a few potential R-genes candidates have been identified, for example *CaETR-1* (*EIN-4 like*) and *ethylene insensitive 3-like gene* (*Ein3*) from the ethylene pathway were mapped in the proximity of the ascochyta blight resistance QTL_{AR1} on LG 4 and QTL_{AR3} on LG 2, respectively (Madrid et al., 2012, 2014). R-gene candidates for ascochyta blight resistance have also been reported based on the gene expression analysis in cultivars with different levels of resistance following *A. rabiei* infection. Using microarray technology, 97 differentially expressed candidate genes were identified from four genotypes and five-time points after *A. rabiei* inoculation (Coram and Pang, 2006). Using real-time quantitative PCR, six out of 15 defense-related genes showed differential expression among ten chickpea genotypes in response to *A. rabiei* infection (Leo et al., 2016). Most recently, using Whole Genome Sequencing (WGS) and Genome-Wide Association Studies (GWAS), a novel candidate gene was reported belonging to the Serine/threonine Receptor-Like Kinase (RLK) class of R-genes (Li et al., 2017).

In the previous chapter, 121 NBS-LRR genes were identified in the chickpea genome. By placing 16 previously reported QTLs for ascochyta blight resistance onto the physical map of the 'CDC Frontier' genome assembly, 30 NBS-LRR genes were found co-localized with the nine ascochyta blight resistance QTLs. Expression analysis of these co-localized NBS-LRR genes using real-time quantitative PCR showed that 27 NBS-LRR genes had differential expression at least in one genotype and at one time-point upon *A. rabiei* infection and could thus be considered candidate genes for ascochyta blight resistance.

The first objective of this study was to map the QTLs for ascochyta blight resistance in three RIL populations derived from crosses between a susceptible parent 'ICCV 96029' and moderately resistance parents 'Amit', 'CDC Luna' and 'CDC Corinne'. The second objective was to compare the QTLs identified across the RIL populations with the previously reported QTLs to identify common and potentially new genomic regions by anchoring these QTLs on to the physical map of 'CDC Frontier'. The final objective was to identify NBS-LRR genes in

these genomic regions and genetically map them.

4.2 MATERIAL AND METHODS

4.2.1 PLANT MATERIAL

Three recombinant inbred populations were developed at the Crop Development Centre, University of Saskatchewan for dissecting the inheritance of ascochyta blight resistance (Anbessa et al., 2009; Tar'an et al., 2007). Chickpea cultivars 'ICCV 96029', 'Amit', 'CDC Luna' and 'CDC Corinne' were selected as parents to develop these RIL populations. 'ICCV 96029' is a highly susceptible cultivar to ascochyta blight and the other three cultivars are moderately resistant to the disease. 'Amit' and 'CDC Luna' are kabuli type chickpea cultivars, and 'CDC Corinne' and 'ICCV 96029' are desi type chickpea cultivars. The RIL populations were derived by crossing susceptible cultivar 'ICCV 96029" as the female parent with each of the moderately resistant cultivar i.e. 'Amit', 'CDC Luna' and 'CDC Corinne' and were named CPR-02, CPR-03, and CPR-04, respectively, and advanced to F_{10} generations in the greenhouse using the single seed descent method (**Table 4.1**). Daba et al. (2016) reported QTL mapping in the CPR-01, a RIL population derived from the cross between 'CDC Frontier' and 'ICCV 96029' and reported nine QTLs for ascochyta blight resistance on all LGs, except on LG 5, using SNP markers under field and greenhouse conditions. In this study, the QTLs reported by Daba et al. (2016) were scanned for co-localization with NBS-LRR genes followed by mapping of the co-localized NBS-LRR genes in the CPR-01 population.

Table 4.1. Details of three chickpea recombinant inbred line (RIL) populations.

RIL Population	Pedigree	Generation	Population Size
CPR-02	'ICCV 96029' × 'Amit'	$F_{9:10}$	133
CPR-03	'ICCV 96029' × 'CDC Luna'	$F_{9:10}$	137
CPR-04	'ICCV 96029' × 'CDC Corinne'	$F_{9:10}$	87

4.2.2 ASCOCHYTA BLIGHT SCREENING UNDER GREENHOUSE CONDITIONS

CPR-02 and CPR-04 were evaluated for their reaction to ascochyta blight under greenhouse conditions in three separate experiments referred to as 'repeats', with three replications within each repeat, whereas CPR-03 was evaluated in two repeats. Each RIL population was arranged in a completely randomized design. Every RIL was grown at two seeds per pot together with two seeds of the susceptible check cultivar 'ICCV 96029' in a 10 cm^2 pot filled

with Sunshine growing mix #4 (SunGro Horticulture Canada, Vancouver British Columbia, Canada) after manual scarification. One week after seedling emergence, all pots were thinned to one plant per genotype in each pot. Greenhouse conditions were maintained at 22/16°C (day/night) with 16 h photoperiod with additional artificial light.

Three-week-old plants at the ten node stage were inoculated with a monoconidial suspension of *A. rabiei* isolate AR-170. This isolate was selected among 98 isolates collected from across Saskatchewan, Canada, based on its moderate aggressiveness (Vail and Banniza, 2008). Extremely aggressive isolates are not preferred for ascochyta blight evaluation due to lack of complete resistance in chickpea germplasm and because isolates with intermediate aggressiveness are better in discriminating genotypes with quantitative resistance (Armstrong-Cho et al., 2015). Cultures of AR-170 were maintained on oatmeal agar (30 g blended quick oats [Quaker Oats Co., Ontario, Canada], 8.8 g agar [Difco, New Jersey, USA], 100 mg chloramphenicol, 1 L deionized water) for 10 days at 22 °C under incandescent lighting with a 12 h photoperiod. Using a haemocytometer, conidial suspensions with a final concentration of 2×10^5 conidia/mL were prepared and 0.1% of Tween 20 (polyoxyethylene sorbitan monolaurate) was added to the final volume as a surfactant.

Approximately 3 mL of suspension was applied to each pot containing the RIL and check plant using an air compressor. After inoculation, all pots were wrapped with clear plastic to maintain high humidity and to avoid contact with adjacent plants. The inoculated plants were moved into the humidity chamber for 48 h and were then moved to greenhouse benches outfitted with an overhead misting system generating a fine mist for 20 seconds after every 60 minutes. Inoculated plants were maintained at 20°C during the day and 18°C during the night and 16 h photoperiod for the rest of the experiment. The first ascochyta blight (AB1) disease rating was conducted two weeks after inoculation using a rating scale of 0 to 9 (Chongo et al., 2004). Two subsequent ratings (AB2 and AB3) were done at weekly intervals. Only AB2 disease ratings were used for analysis of variance (ANOVA) and QTL mapping.

4.2.3 ASCOCHYTA BLIGHT SCREENING UNDER FIELD CONDITIONS

For phenotypic assessment of ascochyta blight under field conditions, all three RIL populations along with their parents were grown at Elrose (51° 01’ 34” N, 108° 03’22” W), Saskatchewan, Canada, in 2014 and 2015. The RILs were grown in 1 m × 1 m microplots and were arranged in a randomized complete block design with three replications. Forty seeds of each RIL were grown per plot. No artificial inoculation was applied to the RILs in the field

due to the presence of sufficient natural air-borne *A. rabiei* ascospores. Disease symptoms were assessed three times, during flowering, pod- filling and maturity stages, but only the second disease score (AB2) collected at the pod-filling stage was used for analysis of variance (ANOVA) and for QTL mapping as the parents of the RIL populations showed maximum phenotypic variation at this stage. A disease score was given to each line based on the overall disease development in the microplot using the 0 to 9 scale (Chongo et al., 2004).

4.2.4 PHENOTYPIC DATA ANALYSIS

Statistical analysis was conducted on the disease scores to assess significant differences in the mean ascochyta blight score of individual RIL of each population using SAS software (version 9.4, SAS Institute Inc., Cary, North Carolina, USA). A combined analysis was conducted for disease data collected in the greenhouse from the three repeats of CPR-02 and CPR-04 RIL populations and two repeats of CPR-03. Homogeneity of variance among repeats and replications was tested using Levene's test and repeated statement was used in PROC MIXED to model heterogeneous variance if required. Analysis of variance (ANOVA) was conducted using PROC MIXED in which genotype was considered a fixed factor and repeats were considered as a random factor. The LSMEANS statement was used to compute the estimate of ascochyta blight scores for each RIL within a population.

For field disease evaluations, combined analysis for 2014 and 2015 and separate analysis of each year were conducted. In the combined analysis, homogeneity of variance among years and blocks was tested using Levene's test and the repeated statement was used in PROC MIXED to model heterogeneous variance if required. ANOVA was conducted using PROC MIXED in which genotype was considered as a fixed factor, year and blocks nested within year were random factors. The LSMEANS statement was used to compute the estimates of ascochyta blight score for each RIL. For separate analysis of each year, ANOVA was calculated using PROC MIXED, in which each RIL was considered a fixed factor and block was considered a random factor.

4.2.5 GENETIC LINKAGE MAP CONSTRUCTION

Genetic linkage map construction of each population was based on the SNP dataset generated through Genotyping by Sequencing (GBS) and Illumina GoldenGate assay. Alleles were defined in parents for each marker, as allele 'A' for 'ICCV 96029' in all three populations and allele 'B' for 'Amit' in CPR-02, 'CDC Luna" in CPR-03, 'CDC Corinne' in CPR-04 in the data matrix used for the construction of linkage maps. Linkage maps were constructed

using Icimapping v3.2 software (http://www.isbreeding.net/). Markers were grouped by LOD scores of 4 to 6, marker order was determined using RECORD algorithm and rippling was done with the COUNT criterion with a window size of 5 markers.

4.2.6 QTL ANALYSES

QTL analyses were conducted using the estimates of the second ascochyta blight disease rating (AB2) under greenhouse and two years (2014 and 2015) of field evaluations, generated through the LSMEANS statement. The analyses were conducted using the Windows QTL Cartographer v2.5 software (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm ; Wang et al., 2012). To locate putative QTLs, the Composite Interval Mapping (CIM) procedure was conducted using Model 6, a standard model with forward and backward regression method, window size of 10 cM and walking speed of 1cM. The LOD score threshold for detection of QTL was calculated by 1000 permutations at p-value of 0.05. The Mapchart v2.2 (Voorrips, 2002) software was used to visualize the genetic linkage map and QTLs.

4.2.7 COMMON GENOMIC REGIONS AND CANDIDATE GENES FOR ASCOCHYTA BLIGHT RESISTANCE

In order to identify common genomic regions and candidate genes (primarily NBS-LRR genes), QTLs identified in CPR-02, CPR-03,CPR-04 and QTLs identified in population CPR-01 by Daba et al. (2016) and all previously reported QTLs were anchored to the physical map of 'CDC Frontier'. All previously reported QTLs and their linked marker sequence information was gathered from the public domain database (https://www.coolseasonfoodlegume.org) and their physical positions were retrieved using sequence alignment BlastN. Using the physical position of the corresponding markers, all QTLs were positioned onto the physical map. To identify the co-localized NBS-LRR genes within the ascochyta blight QTLs, the first LOD confidence interval limit (1-LOD) was used for each QTL identified in CPR-02, CPR-03 and CPR-04 and CPR-01. All NBS-LRR genes were positioned onto the physical map and the NBS-LRR genes present within the 1-LOD interval of each QTL were identified.

4.2.8 GENETIC MAPPING OF CO-LOCALIZED NBS-LRR GENES

Among the co-localized NBS-LRR genes within the ascochyta blight QTLs, polymorphic sequences of the genes between the parents of each RIL population were identified using whole genome resequencing data. KASP markers were designed for the selected polymorphic

NBS-LRR genes using KASP by Design (KBD) software. Genomic DNA from the RILs was used in combination with KASP assay mix (primers) and High Rox KASP master mix for genotyping the RILs using Kompetitive Allele Specific PCR (KASP) assay on an ABI Stepone qPCR instrument. Results were visualized using the STEPone software. For each gene, the alleles of parent 1 and parent 2 were encoded as A and B, respectively, in the data generated from KASP genotyping. By combining the genotypic data generated from each gene and the previous genotypic data from each RIL, a new genetic linkage map was constructed using IcimappingV3.2 software (http://www.isbreeding.net/). Markers were grouped by LOD scores of 4 to 6, marker order was determined using the RECORD algorithm and rippling was done with COUNT criterion with a window size of 5 markers. The final linkage map of each RIL was constructed using the combination of all SNPs including the new NBS-LRR gene markers. QTL analysis was conducted again using the new linkage map and the previous phenotypic data of each RIL population.

4.3 RESULTS

4.3.1 PHENOTYPIC EVALUATION

All three populations, CPR-02, CPR-03 and CPR-04, showed normal frequency distributions and a range of variation for their reaction to ascochyta blight at the second disease rating (AB2) conducted at flowering stage in the experimental repeats under greenhouse conditions and for AB2 disease rating conducted at the pod-filling stage under field condition at Elrose in 2014 and 2015 (**Figure 4.1, Table 4.2**). Under greenhouse conditions, the mean ascochyta blight scores for CPR-02, CPR-03, and CPR-04 were 5.0, 5.6 and 5.3, respectively. The data from the three experimental repeats of CPR-02 and CPR-04 and two repeats of CPR-03 under greenhouse conditions were combined for ANOVA and the overall mean of ascochyta blight scores were used for QTL analysis. The ANOVA showed that the genotypes (RILs) had a significant effect on ascochyta blight severity at $P < 0.001$ in all three populations.

Under field conditions at Elrose in 2014 and 2015, the mean ascochyta blight scores for CPR-02, CPR-03, and CPR-04 were 5.4, 6.0, and 5.7, respectively. Environment plays an important role in disease development and progression under field conditions. The differences in annual weather conditions may have affected disease development, therefore, both combined and individual year ANOVA were conducted for field data. Results showed that the RILs had a significant effect at $P < 0.001$ on ascochyta blight severity in all three

populations in both combined and individual analyses in all three populations (**Table 4.3)**. There was a significant effect of environment (year) and genotype by environment (year) interaction in CPR-02 and CPR-04. In CPR-03, the effect of year was significant, but the genotype by year interaction was not significant. Therefore, mean estimates for QTL analysis of CPR-03 data were calculated from combined data of 2014 and 2015, whereas for CPR-02 and CPR-04 the QTL analyses were done using data from individual years.

Table 4.2. Arithmetic mean with standard deviations (SD), range and C.V values of the three RIL populations evaluated for ascochyta blight severity in the greenhouse in three repeated experiments and under field conditions in 2014 and 2015 at Elrose, SK.

		CPR-02			CPR-03			CPR-04		
Location	**Level**	**Mean ± SD**	**Range**	**C.V (%)**	**Mean ± SD**	**Range**	**C.V (%)**	**Mean ± SD**	**Range**	**C.V (%)**
Greenhouse	Repeat 1	5.1 ± 1.3	2.0─8.0	20.8	5.5 ± 1.2	3─8	20.4	5.6 ± 1.5	2─9	26.3
	Repeat 2	5.1 ± 1.1	3.0─8.0	15.4	5.6 ± 1.2	3─8	21.4	5.1 ± 1.2	3─8	24.2
	Repeat 3	5.0 ± 1.1	3.0─8.0	14.7	-	-	-	5.1 ± 1.1	3─8	23.0
Elrose	2014	5.3 ± 0.9	4.0─7.0	13.2	5.8 ± 1.1	3─8	20.4	5.1 ± 0.9	4─8	17.1
Elrose	2015	5.5 ± 1.0	3.0─8.5	18.4	6.2 ± 1.2	3─9	18.5	6.2 ± 1	3─8.5	17.6

Note: C.V= Coefficient of variation, Repeat 1, 2 and 3 are individual experiment repeats with three replications each.

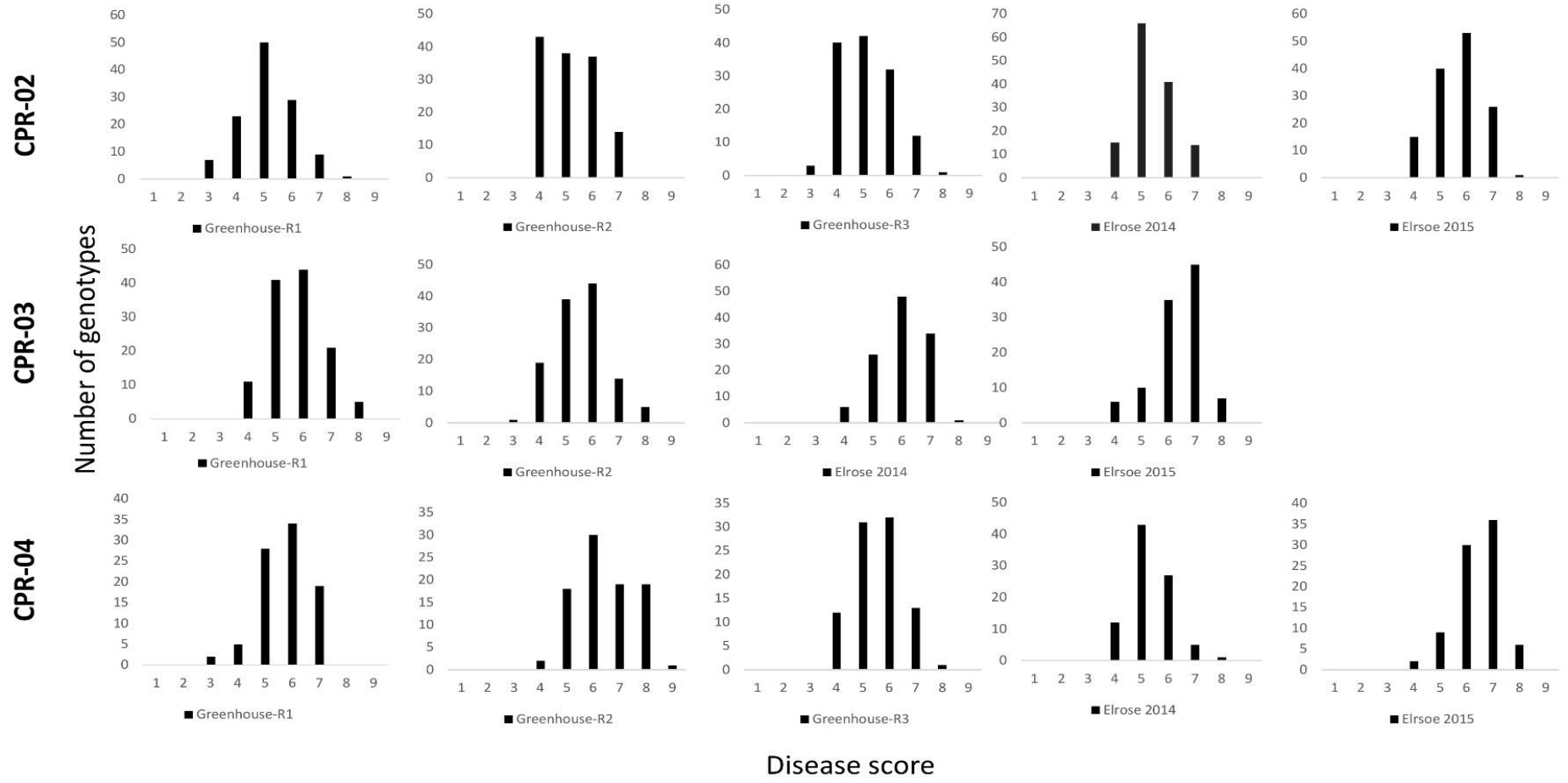

Figure 4.1. Frequency distributions of ascochyta blight severity (flowering stage in the greenhouse and podding stage in the field) in RIL populations CPR-02 ('ICCV 96029/Amit'), CPR-03('ICCV 96029/CDC Luna') and CPR-04 ('ICCV 96029/CDC Corinne') under greenhouse conditions in three experimental repeats (R1; repeat 1, R2; repeat 2 and R3; repeat 3) and under field conditions at Elrose, SK, in 2014 and 2015.

Table 4.3. Analysis of variance (F-values) for the second ascochyta blight score (flowering stage in the greenhouse and podding stage in the field) in CPR-02, CPR-03 and CPR-04 RIL populations under greenhouse (combined data of three experimental repeats) and field conditions at Elrose, SK, in 2014 and 2015.

Location	Level	Effect	CPR-02	CPR-03	CPR-04
Greenhouse	Combined	G	7.14***	6.75***	6.02***
		R	0.48^{ns}	4.15^{ns}	16.38***
		G x R	0.36^{ns}	0.54^{ns}	1.16^{ns}
Elrose	Combined	G	8.31***	5.61***	6.92***
		Y	75.86***	40.7***	330***
		G x Y	2.74***	0.4^{ns}	3.45***
Elrose	2014	G	5.05***	2.8***	4.29***
Elrose	2015	G	6.43***	5.29***	5.53***

Note: G= genotype which representing each inbred line, R= repeat, Y=year, G x R= genotype by repeat interaction, G x Y= genotype by year interaction, '*', '**' and '***' indicates significant difference at $P \leq 0.05$, 0.01, 0.001 and ns=non-significant.

4.3.2 GENETIC LINKAGE MAP

Over 3,000 SNPs were identified from Genotyping by Sequencing (GBS) and the Illumina GoldenGate array in each of the three populations. The majority of these SNPs were redundant as they were clustered and co-positioned on the same loci on the linkage map, thus, only one SNP per locus was selected and used in the final map. The final linkage maps of CPR-02, CPR-03, and CPR-04 consisted of 446, 321 and 156 SNP markers, respectively, distributed across eight linkage groups which correspond to the eight chickpea chromosomes (**Table 4.4**). The CPR-02 linkage map spanned 832 cM of the chickpea genome with an average marker density of 1.9 cM. In CPR-02, LG 4 and LG 7 were the largest linkage groups (128 cM) with the highest number of markers (88) on LG 4. The CPR-03 linkage map spanned 601 cM of the chickpea genome with an average marker density of 1.9 cM. In CPR-03, LG 7 was the largest linkage group (135 cM) with the highest number of markers (74). The CPR-04 linkage map was comparatively shorter than the other two populations spanning only 505 cM of the chickpea genome with an average marker density of 3.29 cM. In this population, LG 7 was the largest linkage group (90 cM) consisting of 30 marker loci.

Table 4.4. Summary of the genetic maps of three chickpea RIL populations.

Population	Map property	CHR-1	CHR-2	CHR-3	CHR-4	CHR-5	CHR-6	CHR-7	CHR-8	Total
CPR-02	Marker number	54	58	59	88	33	67	64	23	446
	Map length (cM)	114	103	84	128	99	117	128	60	832
	Marker interval (cM)	2.1	1.8	1.4	1.5	3.0	1.7	2.0	2.6	1.9
CPR-03	Marker number	50	38	33	72	9	29	74	16	321
	Map length (cM)	98	95	62	122	29	39	135	21	601
	Marker interval (cM)	2	2.5	1.9	1.7	3.2	1.3	1.8	1.3	1.9
CPR-04	Marker number	16	19	14	30	17	14	30	16	156
	Map length (cM)	52	75	41	87	68	34	98	48	505
	Marker interval (cM)	3.2	3.9	2.9	2.8	4	2.4	3.2	2.9	3.29

Note: CHR: chromosome, cM; centimorgan.

4.3.3 QTL FOR ASCOCHYTA BLIGHT RESISTANCE

In total, 18 QTLs for ascochyta blight resistance were identified on LGs 2, 3, 4, 5, 6 and 8 in CPR-02, CPR-03, and CPR-04 under greenhouse and field conditions (**Table 4.5**). For CPR-02, nine QTLs were identified on LGs 2, 3, 4, 5 and 6 (**Figure 4.2**), four QTLs were identified on LGs 2, 4 and 6 for CPR-03 (**Figure 4.3**) and five QTLs were identified on LGs 2, 5, 6 and 8 for CPR-04 (**Figure 4.4**). At least one QTL was identified on LG 2 and LG 6 of each population.

On LG 2, four QTLs were detected in all three populations. In CPR-02, two QTLs *qAB-2-2.1* and *qAB-2-2.2* were identified for Elrose 2015 and greenhouse data explaining 7.9% and 9.3% of the total phenotypic variation, respectively. In CPR-03, one QTL *qAB-3-2.1* was identified for greenhouse data explaining 8% of the total phenotypic variation. In CPR-04, one QTL *qAB-4-2.1* was identified from Elrose 2015 data explaining 21.5% of the total phenotypic variation.

On LG 3, two QTLs *qAB-2-3.1* and *qAB-2-3.2* were identified in the CPR-02 population, which explained 8.5% and 6% of the total phenotypic variation of greenhouse and Elrose 2015 data, respectively.

On LG 4, a total of four QTLs were identified in two populations; CPR-02 and CPR-03. In CPR-02, two QTLs *qAB-2-4.1* and *qAB-2-4.2* were identified for 2014 and 2015 data from Elrose explaining 20 % and 8.8% of the total phenotypic variation, respectively. In CPR-03, two QTLs *qAB-3-4.1* and *qAB-3-4.2* were identified using combined 2014 and 2015 data from Elrose, which explained 13.7% and 13.3% of the total phenotypic variation, respectively.

On LG 5, four QTLs were identified in CPR-02 and CPR-04. In CPR-02, a QTL *qAB-2-5.1* explaining 18.5% of the total phenotypic variation was identified from 2015 data at Elrose and a major QTL *qAB-2-5.2* was consistently detected using combined data of both years explaining 11% (2014) and 33% (2015) of the total phenotypic variation. In CPR-04, two QTLs *qAB-4-5.1* and *qAB-4-5.2* were identified from Elrose 2015 data, which explained 16.5% and 16.9% of the total phenotypic variation, respectively.

On LG 6, one QTL was detected in each of the three populations. In CPR-02, a QTL *qAB-2-6.1* was identified from greenhouse data explaining 12.2% of the total phenotypic variation. In CPR-03, a QTL *qAB-3-6.1* was identified from combined data collected at Elrose

explaining 12.9% of the total phenotypic variation. In CPR-04, a QTL *qAB-4-6.1* was identified from Elrose 2014 data explaining 10.8% of the total phenotypic variation.

On LG 8, only one QTL *qAB-4-8.1* was identified in CPR-04 population, which was consistent for greenhouse and Elrose 2015 data and explained 24.6% and 11.7% of the total phenotypic variation, respectively.

Table 4.5. Quantitative trait loci (QTLs) identified for ascochyta blight resistance under greenhouse and field conditions in RIL populations CPR-02, CPR-03, and CPR-04.

Linkage Group (LG)	QTL	Population	Environment	Peak Marker	Position (cM)	Additive	%PV	LOD
2	*qAB-2-2.1*	CPR-02	Elrose 2015	TMV1	32.71	0.26	7.9	4.6
	qAB-2-2.2	CPR-02	Greenhouse	ABA-R	44.51	0.25	9.3	3.3
	qAB-3-2.1	CPR-03	Greenhouse	SCA2_5541651	54.41	0.27	8.0	3.0
	qAB-4-2.1	CPR-04	Elrose 2015	SCA2_22447260	25.11	0.44	21.5	3.8
3	*qAB-2-3.1*	CPR-02	Greenhouse	SCA3_34280455	50.11	0.24	8.5	3.2
	qAB-2-3.2	CPR-02	Elrose 2015	SCA3_15444471	82.31	0.22	6.0	3.4
4	*qAB-2-4.1*	CPR-02	Elrose 2014	Cav1sc62.1p168325	38.01	0.40	20.0	8.2
	qAB-2-4.2	CPR-02	Elrose 2015	Cav1sc25.1p1095606	99.41	0.28	8.8	5.1
	qAB-3-4.1	CPR-03	Elrose	SCA4_5006008	42.91	0.30	13.7	5.1
	qAB-3-4.2	CPR-03	Elrose	SCA4_43782809	111.71	0.31	13.3	5.8
5	*qAB-2-5.1*	CPR-02	Elrose 2015	SCA5_9917236	40.41	0.36	18.5	6.6
	qAB-2-5.2	CPR-02	Elrose 2014	SCA5_20906121	51.41	0.29	11.1	4.4
			Elrose 2015	SCA5_20906121	49.41	0.48	33.0	14.4
	qAB-4-5.1	CPR-04	Elrose 2015	SCA5_20906121	9.91	0.38	16.5	4.7
	qAB-4-5.2	CPR-04	Elrose 2015	SCA5_22834238	20.31	0.39	16.9	3.5
6	*qAB-2-6.1*	CPR-02	Greenhouse	Cav1sc30.1p60427	34.61	0.56	12.2	5.4
	qAB-3-6.1	CPR-03	Elrose	Cav1sc445.1p92883	18.11	0.30	12.9	5.7
	qAB-4-6.1	CPR-04	Elrose 2014	SCA6_21016641	3.71	0.26	10.8	3.2
8	*qAB-4-8.1*	CPR-04	Greenhouse	SCA8_8579792	23.91	0.40	24.6	7.0
			Elrose 2015	SCA8_8579792	21.81	0.33	11.7	4.2

Note: In CPR-03; Elrose = combined field data of Elrose 2014 and 2015, each QTL was named as qAB (QTL for Ascochyta blight - RIL # - Linkage group # #. no. of QTL), CPR; Chickpea population RIL, cM; centimorgan, % PV; Percent of phenotypic variation explained, LOD; Log 10 of the odds ratio.

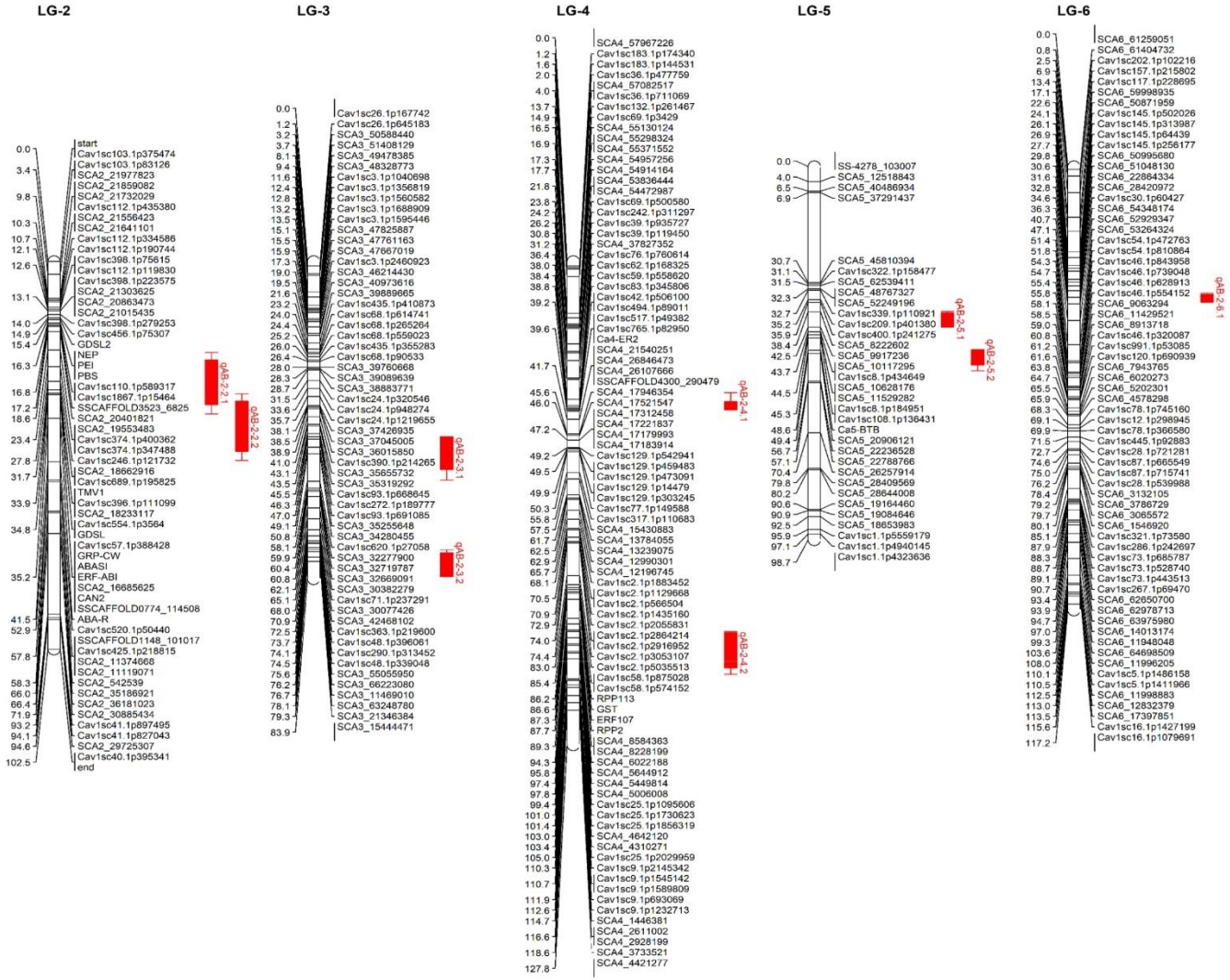

Figure 4.2. QTLs detected for ascochyta blight resistance on linkage groups (LGs) 2, 3, 4, 5 and 6 under greenhouse and field conditions in CPR-02. On LG 5, QTL (*qAB-2-5.2*) was consistently detected for data from Elrose in 2014 and 2015.

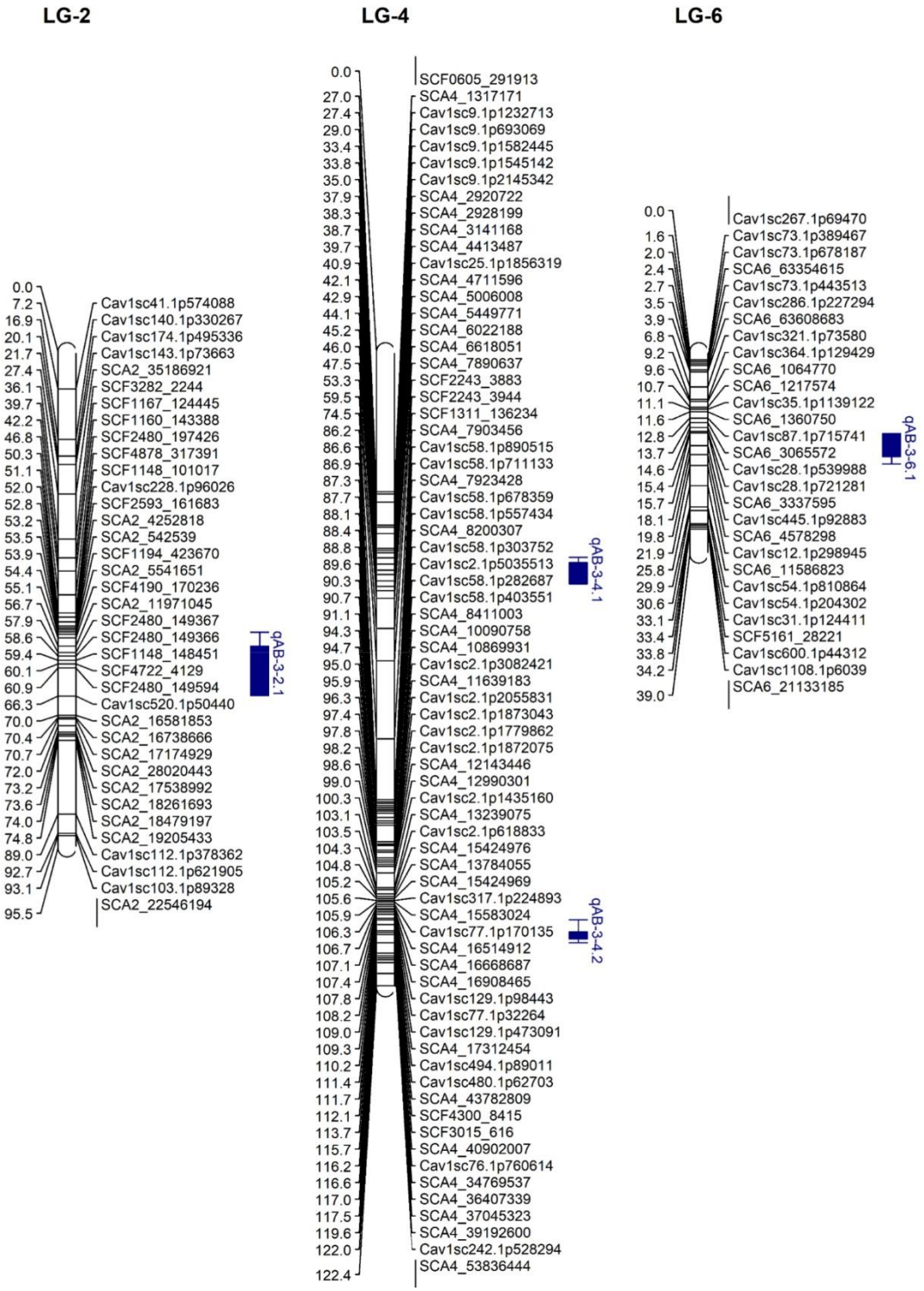

Figure 4.3. QTLs identified for ascochyta blight resistance on linkage groups (LGs) 2, 4 and 6 under greenhouse and field conditions in chickpea RIL population CPR-03. QTL *qAB-3-2.1* was only detected from greenhouse data and the other three QTLs were detected from combined field data of 2014 and 2015.

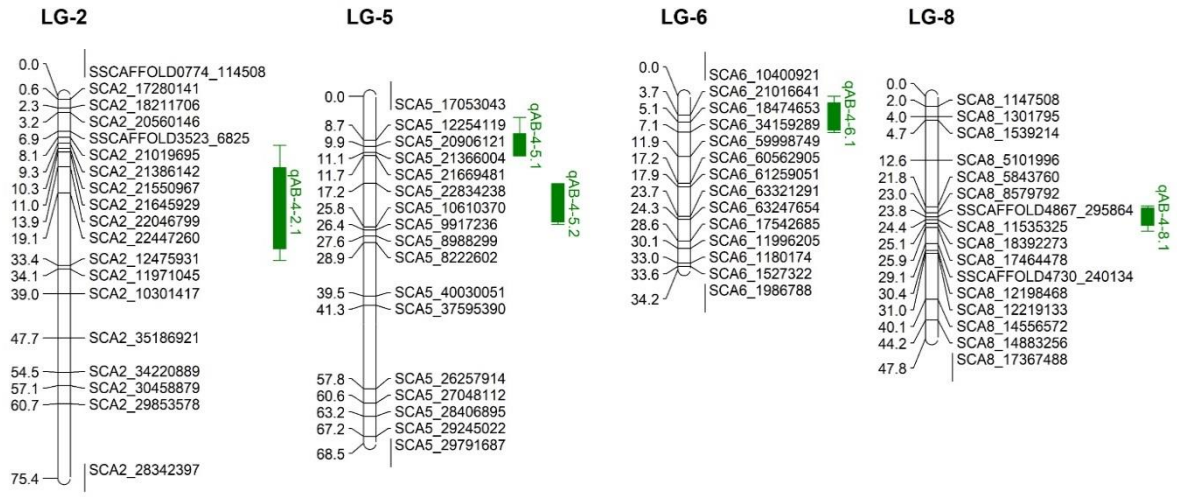

Figure 4.4. QTLs identified for ascochyta blight resistance on linkage groups (LGs) 2, 5, 6 and 8 under greenhouse and field conditions in chickpea population CPR-04. On LG 8, QTL *qAB-4-8.1* was consistently detected using greenhouse and field data from Elrose 2015.

4.3.4 COMMON GENOMIC REGIONS FOR ASCOCHYTA BLIGHT RESISTANCE

To identify common genomic regions for ascochyta blight resistance, 15 previously reported QTLs, nine QTLs identified in CPR-01 population by Daba et al. (2016) and 18 QTLs identified in CPR-02, CPR-03, and CPR-04 populations from the current study were anchored to the physical map of 'CDC Frontier' based on the physical position of their corresponding markers. Most of the QTLs were physically mapped on common genomic regions. Such regions with over-lapping QTLs were found on chromosomes 2, 3, 4, 5, 6 and 8 (**Figure 4.5**).

On chromosome 2, *QTL1* was physically mapped to a 13-35 Mb region. The QTL on LG 2 was initially reported in a F_2 mapping population derived from a cross between 'ICCV 96029/CDC Luna' using markers TR19 and TA110 (Anbessa et al., 2009). *QTL[ar1a]* specific to *A. rabiei* pathotype I was reported using SSR markers GA16 and GA20 in a RIL population derived from a cross between 'PI 359075/FLIP84-92C' and this QTL was also mapped to this 20 Mb region (Cho et al., 2004). Another QTL *qtlAB-1.1* reported by Daba et al. (2016) in the CPR-01 RIL population was positioned in a 15-17 Mb region. Two QTLs *qAB-2-2.1* (greenhouse) and *qAB-2-2.1* (Elrose 2015) identified in the CPR-02 were positioned at 16-19 Mb and 14-16 Mb regions, respectively. In the CPR-03, QTL *qAB-3-2.1* (greenhouse) was positioned at 5-14 Mb and QTL *qAB-4-2.1* (Elrose 2015) in CPR-04 was positioned in the 12-21 Mb region of this chromosome.

On chromosome 3, *QTL2* was physically mapped on 30-37 Mb region which was previously reported on LG 3 in the two F_2 mapping populations 'ICCV 96029/CDC Frontier' and 'ICCV 96029/Amit', using markers TS19-TA64 and TR26-TA64, respectively (Anbessa et al., 2009; Tar'an et al., 2007). From our study, QTL *qAB-2-3.1* (greenhouse) identified in CPR-02 was positioned in the same physical region (at 32-35 Mb of chromosome 3).

On chromosome 4, two major clusters of QTLs were found in 4-15 Mb and 20-43 Mb region of the chromosome. In the 4-8 Mb region, QTL_{AR1} reported by Iruela et al., (2006) and candidate gene *CaETR1* reported by Madrid et al. (2012) were physically mapped. From our study, QTL *qAB-2-4.2* (Elrose 2015) identified in CPR-02 and QTL *qAB-3-4.2* (Elrose 2014-2015 combined) identified in CPR-03 were placed into the 2-5 Mb and 4-5 Mb regions, respectively. Another *A. rabiei* pathotype II specific *QTL[ar2b]* reported by Cho et al. (2004) on chromosome 4 was placed at the 8 Mb region. Seedling resistance QTL *AB-Q-SR-4-1* was positioned near this region (8-15 Mb) based on the position of markers STMS11 and TA130 in a F_2 mapping population 'C 214/ILC 3279' (Sabbavarapu et al., 2013). QTL *qtlAB-4.1* reported by Daba et al. (2016) in CPR-01 was positioned in the same region (6-13 MB). In the 24-43 Mb regions of chromosome 4, QTL_{AR2} reported by Iruela et al., (2006) was physically mapped based on the position of TA2 and TA72 SSR markers. In the same region (24-41 Mb), *QTL2* was physically mapped, which was previously reported on LG 4 based on markers TA2 and TA146 in a F_2 mapping population derived from a cross between 'ICCV 96029/CDC Frontier' (Tar'an et al., 2007). Another seedling resistance QTL, *AB-Q-SR-4-2*, was positioned in the 31-41 Mb region of chromosome 4 based on the position of the H4G11 and CaM2049 markers (Sabbavarapu et al., 2013). From our study, QTL *qAB-2-4.2* (Elrose 2014) identified in CPR-02 was positioned in the 21-37 Mb and QTL *qAB-3-4.2* (Elrose 2014-2015 combined) identified in CPR-03 was positioned on 17-43 Mb regions of chromosome 4.

On chromosome 5, three QTLs from our study were localized in the 10-20 Mb region. QTL *qAB-2-5.2* (Elrose 2014, Elrose 2015) identified in CPR-02 was positioned in the 11-22 Mb region and QTLs *qAB-4-5.1* and *qAB-5-5.2* (Elrose 2015) identified in CPR-03 were positioned in 12-21 Mb and 10-22 Mb regions, respectively, of this chromosome.

On chromosome 6, the two adult plant resistance QTLs *AB-Q-APR-6-1* and *AB-Q-APR-6-2* were positioned in the 1-12 Mb region based on the position of H1T16-TA106 and TA106-CaM0244 markers, respectively (Sabbavarapu et al., 2013). QTL *qtlAB-6.2* reported by Daba

et al. (2016) in CPR-01 was positioned in the 1-2 Mb region. *QTL4* previously reported by Tar'an et al. (2007) on LG 6 using the TA2 and TA146 markers was physically mapped into the 5-53 Mb region of this chromosome. In the same region, QTLs *qAB-2-6.1* (greenhouse) identified in CPR-02 was positioned in the 28-54 Mb region and *qAB-4-4.1* (Elrose 2014) identified in CPR-04 was positioned into the 10-34 Mb region of chromosome 6.

On chromosome 8, Anbessa et al. (2009) reported *QTL5* on LG 8 using a F_2 mapping population derived from a cross between 'ICCV 96029/CDC Corinne' that was physically mapped onto 1-5 Mb region based on the position of the GA6 and TS45 markers. QTL qtlAB-8.1 reported by Daba et al. (2016) in CPR-01 RIL was positioned in the 1-5 Mb region. In our study, QTL *qAB-4-8.1* (greenhouse, Elrose 2015) identified in CPR-04 was placed on the 5-18 Mb region which was placed near *QTL5,* but does not have any over-lapping region. QTLs *qtlAB-8.1* and *qtlAB-8.3* reported by Daba et al. (2016) in the CPR-01 RIL were positioned at 11 Mb and in the 13-14 Mb region, respectively, and overlapped with the physical position of QTL *qAB-4-8.1* identified in CPR-04.

4.3.5 NEW GENOMIC REGIONS FOR ASCOCHYTA BLIGHT RESISTANCE

In the CPR-02 population, a major QTL *qAB-2-5.2* on LG 5 was identified consistently under field condition in 2014 (LOD= 4.4, PVE= 11%) and 2015 (LOD= 14.4, PVE= 33%) at Elrose. In CPR-04, QTLs *qAB-4-5.1* (LOD= 4.7, PVE= 16.5%) and *qAB-4-5.2* (LOD= 3.5, PVE= 16.9%) were also identified on LG 5, but only from Elrose 2015 data. Previously, QTL *AB-Q-APR-5B* was reported on LG 5 by Sabbavarapu et al. (2013) in a F_2 mapping population derived from a cross between 'C 214/ILC 3279'. Anchoring these QTLs onto the physical map based on their marker positions revealed that QTL *qAB-2-5.2* identified in CPR-02 and QTLs *qAB-4-5.1* and *qAB-4-5.2* identified in CPR-04 were present in the same physical location on chromosome 5 and there was no overlap with the previously reported QTL *AB-Q-APR-5B*, indicating that QTLs *qAB-2-5.2, qAB-4-5.1,* and *qAB-4-5.2* are new QTLs for ascochyta blight resistance. Similarly, a new QTL *qAB-2-3.2* was identified on LG 3 in the CPR-02 population under field condition of 2015 (LOD= 3.4, PVE= 6%). Another new QTL (*qAB-4-8.1*) was identified in CPR-04 population under both greenhouse (LOD= 7, PVE= 24.6%) and field conditions in Elrose 2015 (LOD= 4.2, PVE= 11.7%) on LG 8.

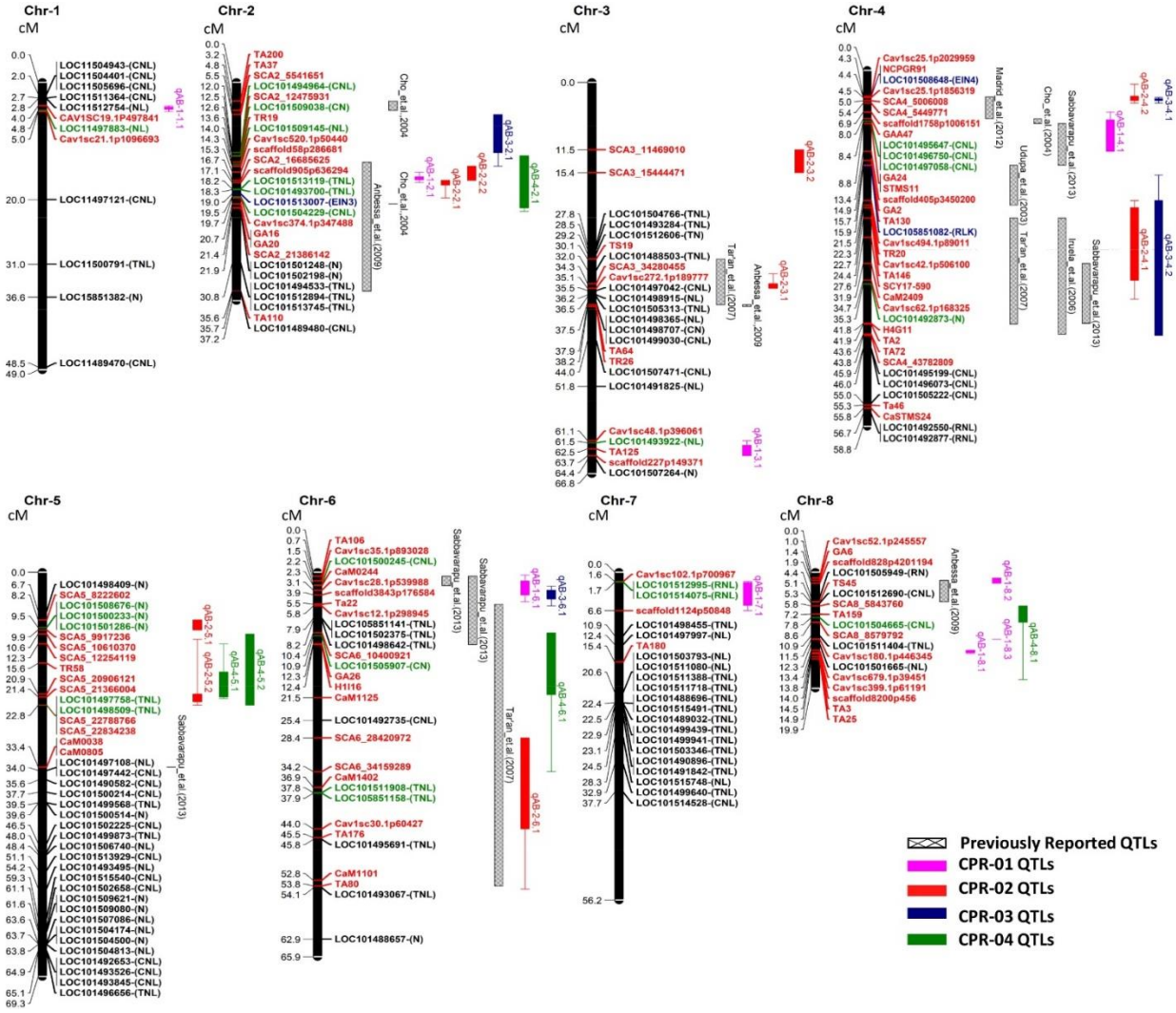

Figure 4.5. The physical map of the CDC Frontier genome assembly along with physical position of NBS-LRR genes (in black). The markers used to anchor QTLs identified in four RIL populations and previously reported QTLs are shown in red. QTLs were color-coded separately as shown in the legend. NBS-LRR genes co-localized with QTLs identified in four RIL populations are presented in green.

4.3.6 CO-LOCALIZED NBS-LRR GENES WITH ASCOCHYTA BLIGHT QTLs

The approach of 1-LOD confidence interval was used to identify the genomic regions containing the co-localized NBS-LRR genes with the ascochyta blight resistance QTLs identified in all three populations from our study, and the QTLs identified in CPR-01 population by Daba et al. (2016). In total, 32 NBS-LRR genes were identified to be co-localized with the physical position of 16 QTLs detected for ascochyta blight resistance in four RIL populations (**Table 4.6)**. In CPR-01, eight NBS-LRR genes were co-localized with five QTLs, 11 NBS-LRR genes were co-localized with five QTLs in CPR-02, four NBS-LRR genes were co-localized with two QTLs in CPR-03 and nine NBS-LRR genes were co-localized with four QTLs in CPR-04. Among these 32 co-localized genes, most of the NBS-LRR genes belong to the TNL class (10), followed by CNL (8), NBS (5), NL (4) CN (3) and RNL (2). Most of the co-localized NBS-LRR genes are present as singletons, but a few clusters of NBS-LRR genes were also found. In CPR-01, a cluster of three CNL genes were co-localized with QTL *qAB-1-4.1* on chromosome 4 and a cluster of two RNL class genes in QTL *qAB-1-7.1* on chromosome 7. In CPR-02, a cluster of three NBS genes were co-localized with QTL *qAB-2-5.1* and a cluster of two TNL class genes in QTL *qAB-2-5.2* on chromosome 5. Common NBS-LRR genes were identified within the QTLs from different RIL populations on the same chromosome with over-lapping regions. Eight co-localized NBS-LRR genes that were common in at least two populations were identified. On chromosome 2, three NBS-LRR genes (*LOC101503119*, *LOC101493700*, and *LOC101504229*) were co-localized with QTLs *qAB-2-2.1* and *qAB-4-2.1*, respectively, in CPR-02 and CPR-04, and two NBS-LRR genes (*LOC101509038* and *LOC101509145*) were co-localized in QTLs *qAB-3-2.1* and *qAB-4-2.1* in CPR-03 and CPR-04, respectively. On chromosome 4, a NBS class gene *LOC101492873* was co-localized with QTLs *qAB-2-4.1* and *qAB-3-2.1* in CPR-02 and CPR-03, respectively. On chromosome 5, a cluster of two TNL class NBS-LRR genes (*LOC101497758* and *LOC101498509*) co-localized with QTLs *qAB-2-5.2* and *qAB-4-5.1* in CPR-02 and CPR-04, respectively.

Table 4.6. Candidate NBS-LRR genes co-locating with QTLs detected for ascochyta blight resistance in four chickpea RIL populations.

Population	QTL	Chr.	QTL interval (bp)	Gene ID	Class
CPR-01	*qAB-1.1*	1	4023853-4988933	LOC101497883	NL
	qAB-2.1	2	15270543-17080573	-	-
	qAB-3.1	3	61124446-63730491	LOC101493922	NL
	qAB-4.1	4	6907594-13443128	LOC101495647	CNL
				LOC101496750	CNL
				LOC101497058	CNL
	qAB-6.1	6	1453293-3896138	LOC101500245	CNL
	qAB-7.1	7	1629328-6561261	LOC101512995	RNL
				LOC101514075	RNL
	qAB-8.1	8	13356114-13972043	-	-
	qAB-8.2	8	1015596-1949215	-	-
	qAB-8.3	8	-	-	-
CPR-02	*qAB-2-2.1*	2	16685625-19721215	LOC101513119	TNL
				LOC101493700	TNL
				LOC101504229	CNL
	qAB-2-2.2	2	14258530-16685625	-	-
	qAB-2-3.1	3	11469010-15444471	-	-
	qAB-2-3.2	3	34280455-35093777	-	-
	qAB-2-4.1	4	22688416-34746488	LOC101492873	N
	qAB-2-4.2	4	4328983-5006008	-	-
	qAB-2-5.1	5	8222602-9917236	LOC101508676	N
				LOC101500233	N
				LOC101501286	N
	qAB-2-5.2	5	20906121-22788766	LOC101497758	TNL
				LOC101498509	TNL
	qAB-2-6.1	6	28420972-54348174	LOC101511908	TNL
				LOC105851158	TNL
CPR-03	*qAB-3-2.1*	2	5541651-14258530	LOC101494964	CNL
				LOC101509038	CN
				LOC101509145	NL
	qAB-3-4.1	4	4497942-5449771	-	-
	qAB-3-4.2	4	21461931-43782809	LOC101492873	N
	qAB-3-6.1	6	3132045-5785634	-	-

Table 4.6. (Continued).

Population	QTL	Chr.	QTL interval (bp)	Gene ID	Class
CPR-04	*qAB-4-2.1*	2	12475931-22046799	LOC101509038	CN
				LOC101509145	NL
				LOC101513119	TNL
				LOC101493700	TNL
				LOC101504229	CNL
	qAB-4-5.1	5	10610370-22834238	LOC101497758	TNL
				LOC101498509	TNL
	qAB-4-5.2	5	12254119-21366004	-	-
	qAB-4-6.1	6	10400921-21016641	LOC101505907	CN
	qAB-4-8.1	8	5843760-8579792	LOC101504665	CNL

4.3.7 GENETIC MAPPING OF CO-LOCALIZED NBS-LRR GENES

Different research groups have identified major QTLs on LG 2 and LG 4 using STMS markers. Millan et al. (2006) concluded that the resistance to *A. rabiei* pathotype I is governed by a major QTL on LG 2 close to marker GA16, and a QTL flanked by STMS 11 and TR20 on LG 4 is responsible for resistance to pathotype II. Physical mapping of ascochyta blight resistance QTLs revealed that the marker GA16 was physically positioned at 20.7 Mb on chromosome 2. The previously reported *QTL1* (Anbessa et al., 2009), *QTL ar1a* specific to pathotype I (Cho et al., 2004), candidate gene *Ein3* (Madrid et al., 2014) and QTLs *qAB-2-2.1* and *qAB-4-2.1* from the current study were all located in this region. Three NBS-LRR genes (*LOC101503119, LOC101493700* and *LOC101504229*) were co-localized within the QTLs *qAB-2-2.1* (CPR-02) and *qAB-4-2.1* (CPR-04). Among these three genes, a single base pair polymorphism between the parents of CPR-02 ('ICCV 96029' and 'Amit') was identified in the TNL gene *LOC101493700*. Similarly, STMS11 (8.8 Mb) and TR20 markers (22.3 Mb) were physically mapped on chromosome 4 that harboured the previously reported *QTL [ar2b]* specific to *A. rabiei* pathotype II (Cho et al., 2004), QTL *AB-Q-SR-4-1* in both seedling and adult plant stages (Sabbavarapu et al., 2013), and QTL *qtlAB-4.1* in CPR-01 as reported by Daba et al. (2016). A cluster of three CNL class NBS-LRR genes (*LOC101497883, LOC101495647, LOC101496750*) on chromosome 4 was co-localized with the QTL *qAB-1-4.1* in CPR-01. Among these three genes, *LOC101495647* and *LOC101496750* were polymorphic between the CPR-01 parents ('ICCV 96029' and 'CDC Frontier'). The NBS-LRR genes *LOC101495647* and *LOC101496750* polymorphic between

the ‘CDC Frontier’ and ‘ICCV 96029’ and *LOC101493700* polymorphic between ‘Amit’ and ‘ICCV 96029’ were selected for genetic mapping in CPR-01 and CPR-02, respectively, to validate the physical co-localization of the NBS-LRR genes in the QTL intervals. New linkage maps were constructed with the addition of these NBS-LRR genes and further QTL analysis was conducted using the new linkage maps. In the new linkage map of CPR-01, the CNL class genes *LOC101495647* and *LOC101496750* were genetically mapped at 21.7 cM in the QTL *qAB-1-4.1* interval of 19-29 cM on LG 4. The LOD score peaked at the exact position of the NBS-LRR genes and the QTL explained 19.6% of the total phenotypic variation (**Figure 4.6**). Similarly, in CPR-02, the TNL class gene *LOC101493700* was genetically mapped at 32.71 cM co-localized within the interval of QTL *qAB-2-2.1* (26-37 cM), which explained 7.9% of the phenotypic variation. Here again, the LOD score peaked at this gene location (**Figure 4.7**).

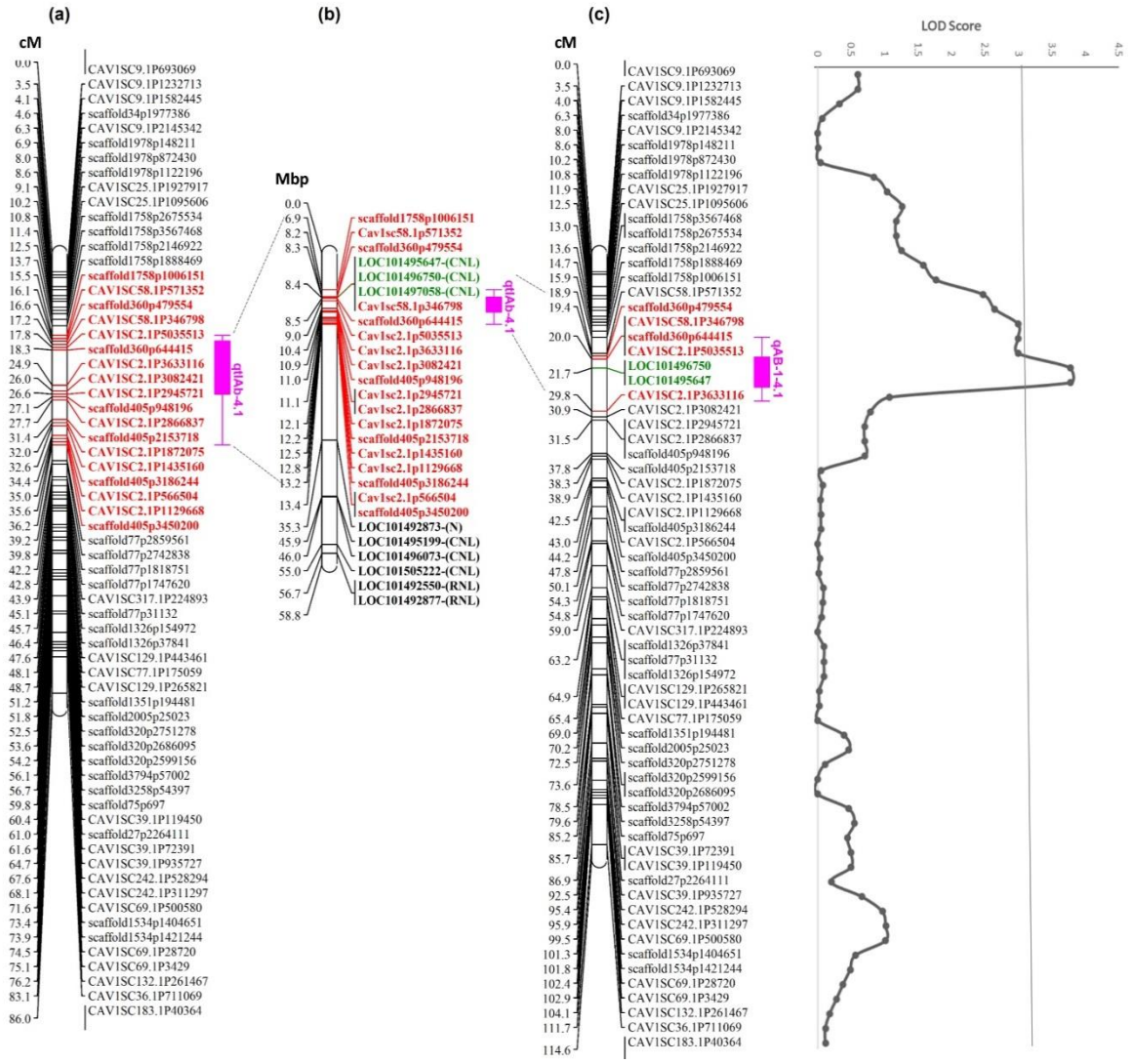

Figure 4.6. The genetic and physical maps of chromosome 4 in CPR-01. (a) The previous genetic map of CPR-01 chromosome 4 in which QTL *qtlAb-4.1* was identified by Daba et al. (2016). All markers in the QTL interval are in red and QTLs are represented as pink bars. (b) The physical map of chickpea chromosome 4, all markers in QTL intervals were mapped to their physical positions and three NBS-LRR genes were found co-localized in the QTL interval. Co-localized genes are presented in green. (c) The new genetic map of CPR-01 chromosome 4 after addition of two SNP markers from NBS-LRR genes. The QTL analysis with the addition of the NBS-LRR genes detected the same QTL (renamed *qAB-1-4.1*) which peaked at the physical location of NBS-LRR genes.

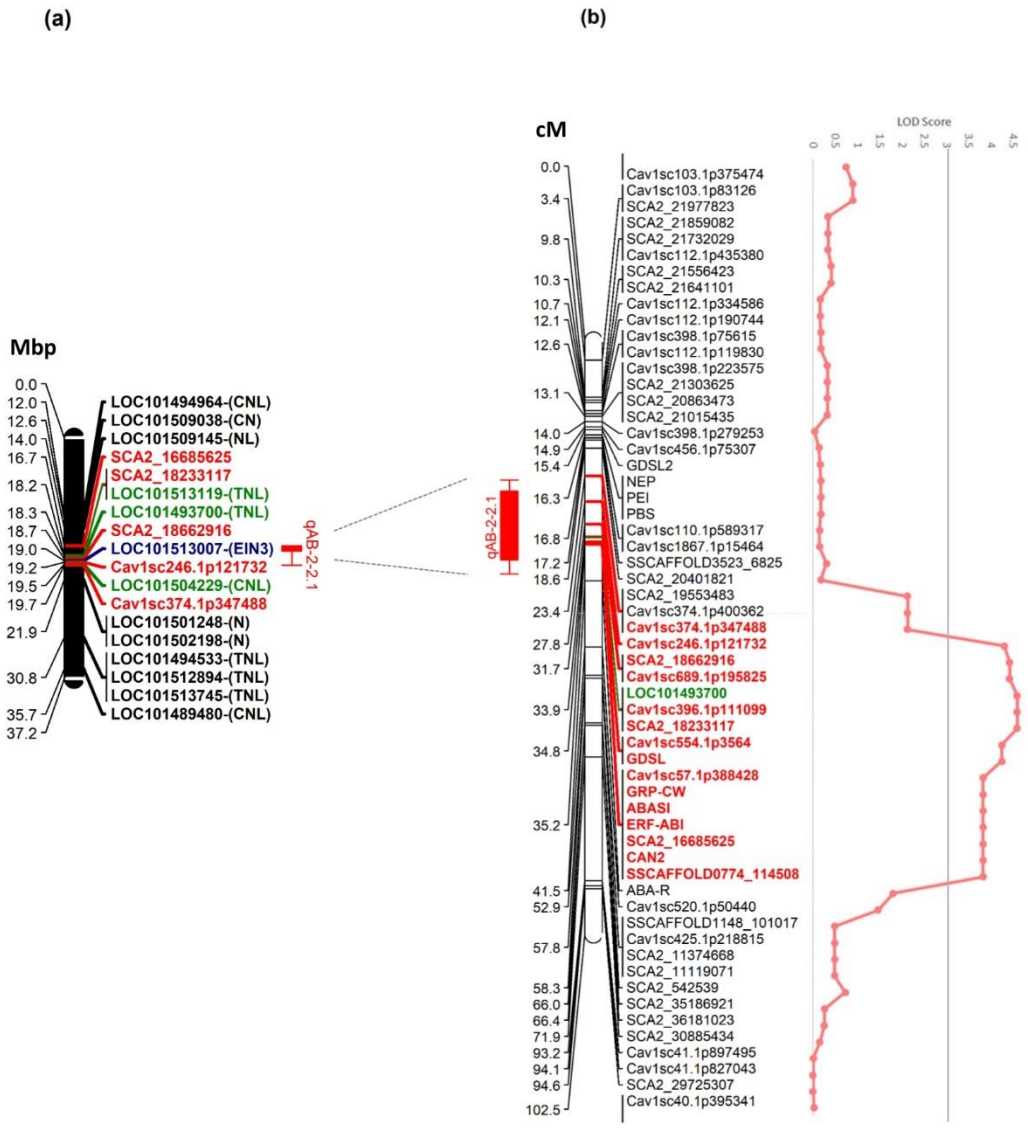

Figure 4.7. The physical and genetic maps of chromosome 2 in CPR-02. (a) The physical map of chickpea chromosome 2 (black bar) along with physical position of NBS-LRR genes (in black), QTL *qAB-2-2.1* was physically mapped onto chromosome 2 based on the SNP markers (in red) and three NBS-LRR genes (in green) were found co-localized in this QTL interval. Co-localized genes are presented in green. (b) The graphical representation of the genetic mapping of TIR-NBS-LRR class gene *LOC101493700* co-localized with QTL qAB-2-2.1 identified in data from Elrose in 2015 on chromosome 2.

4.4 DISCUSSION

Chickpea reactions to ascochyta blight are highly dependent on environmental conditions and other factors such as the type of inoculum, inoculum concentration and plant growth stage (Tivoli et al., 2006). Three ascochyta blight disease ratings were conducted under greenhouse conditions, starting two weeks after inoculation with a monoconidial suspension of an *A. rabiei* isolate in two to three experimental repeats for each RIL population using a semi-quantitative 0-9 disease score scale (Chongo et al., 2004). Under field conditions, disease screening was conducted in 2014 and 2015 at Elrose, SK where the RILs were exposed to natural inoculum and were assessed at three plant growth stages including flowering, pod-filling and maturity. Only disease ratings taken when most of the RILs in each population were at the flowering stage in the greenhouse and the podding stage in the field were used for analyses. The greenhouse datasets from the three repeats were combined because of uniform conditions among the repeats, whereas field data from two years were kept separate for populations CPR-02 and CPR-04 based on significant genotype by environment interactions. However, in CPR-03 this interaction was non-significant, so field data from both years were combined. Significant variation in reactions to ascochyta blight was observed among the RILs under greenhouse and field conditions for each population.

Greenhouse and field data were not combined because of several factors including, single isolate inoculation in the greenhouse *versus* mixed populations of *A. rabiei* in the field, different physiological plant growth stage (flowering *versus* podding) and most importantly due to the significant effect of genotype by environment (greenhouse and field condition) interactions. Significant year by RIL interaction was also observed by Iruela et al. (2006). The difference in chickpea reaction to ascochyta blight disease ratings under controlled environment and field conditions, with higher disease scores in the greenhouse than in the field could be due to more favorable conditions for the disease in the greenhouse (Anbessa et al., 2009).

In total, 18 QTLs for ascochyta blight resistance were identified on LGs 2, 3, 4, 5, 6 and 8 accounting for 6 to 33% of the phenotypic variation in three different populations under greenhouse and field conditions. Some QTLs were location-specific, whereas others were common among the three RIL populations. For example, QTL *qAB-4-8.1* was consistently detected under greenhouse and field conditions, QTL *qAB-2-5.2* was consistent under field conditions in both years, but many other QTLs were specific to either the greenhouse or the year at Elrose. The reason for environment-specific QTLs is primarily the high dependency

of ascochyta blight development on environmental conditions. Similar to our study, both common and different sets of QTLs for ascochyta blight resistance were identified under controlled and field conditions by Sabbavarapu et al. (2013).

In the absence of a consensus map, the physical map of the 'CDC Frontier' chickpea genome assembly was used and all identified QTLs for ascochyta blight resistance from our study and previous studies based on the physical position of their corresponding markers were anchored onto the chickpea physical map. Most of the QTLs were physically mapped on common genomic regions on chromosome 2, 3, 4, 5, 6 and 8 in spite of the fact these QTLs were mapped using different resistant sources under different environmental conditions and using different screening methods. However, a few new QTLs were also identified in this study, such as QTL *qAB-2-5.2* in CPR-02, and QTLs *qAB-4-5.1* and *qAB-4-5.2* in CPR-04 on chromosome 5.

In total, 32 NBS-LRR genes co-localized with the physical position of 16 QTLs associated with ascochyta blight resistance were identified in four RIL populations. Co-localized genes belong to different classes of NBS-LRR genes including TNL, CNL, RNL, NL, CN and NBS. Previous studies have reported the TNL and CNL gene classes to provide resistance against necrotrophic pathogens, e.g. *A. thaliana* TNL class *RLM3* resistance against *B. cinerea*, *A. brassicicola* and *A. brassicas,* and one hemibiotrophic fungus *L. maculans* (Staal et al., 2008). In wheat, over-expression of the *TaRCR1*, a member of the CNL class increased the resistance against the necrotrophic fungus *Rhizoctonia cerealis* (Zhu et al., 2016). In contrast, genes of the RNL class are known to provide resistance against biotrophic pathogens such as the *A. thaliana ADR1* gene which belongs to the RNL class. The expression of this gene elevates the levels of salicylic acid (SA) and reactive oxygen intermediates and provides broad resistance against the biotrophic pathogens *P. parasitica* and *E. cichoracea* (Grant et al., 2003). Most of the co-localized NBS-LRR genes are present as singletons, but a few clusters of NBS-LRR genes were also found co-localized with ascochyta blight resistance QTLs. A few studies have suggested that clusters of the NBS-LRR genes provide effective resistance. A cluster of two NBS-LRR in *Oryza sativa* acted in tandem to provide resistance against *Magnaporthe grisea* and in *A. thaliana* two TNL class genes were required to provide effective resistance against *P. parastica* (Ashikawa et al., 2008; Sinapidou et al., 2004). Therefore, the cluster of NBS-LRR genes co-localized with ascochyta blight resistant QTLs may have a role in providing resistance to *A. rabiei*. Further

functional characterization of such genes is required to confirm and understand their function in resistance against ascochyta blight in chickpea.

Previously, several QTLs were reported on LG 2 and LG 4 using different genetic backgrounds and common STMS markers. In a review by Millan et al. (2006), it was concluded that a QTL on LG 2 close to marker GA16 governs resistance *to A. rabiei* pathotype I and QTL flanked by STMS 11 and TR20 on LG 4 controlled the resistance *to A. rabiei* pathotype II. Considering the importance of these genomic regions, three NBS-LRR genes (*LOC101503119*, *LOC101493700*, *LOC101504229*) co-localizing with QTLs *qAB-2-2.1* (CPR-02) and *qAB-4-2.1* (CPR-04) and the cluster of CNL class genes *LOC101497883*, *LOC101495647* and *LOC101496750* on chromosome 4 co-localizing with QTL qAB-1-4.1 in CPR-01 were selected for genetic mapping. These three CNL genes showed high sequence similarity (e-value=1e-56) with the *A. thaliana RPP13* that provides resistance to powdery mildew (Bittner-Eddy and Beynon, 2001). Based on the polymorphisms between the parents of the RILs, only NBS-LRR genes *LOC101495647* and *LOC101496750* on chromosome 4 in CPR-01 and *LOC101493700* on chromosome 2 in CPR-02 were used for genetic mapping to validate their co-localization. Genetic mapping of CNL class genes *LOC101495647* and *LOC101496750* in *qAB-1-4.1* in CPR-01 and TNL class gene *LOC101493700* in *qAB-2-2.1* in CPR-02 confirmed the physical co-localization of these NBS-LRR genes with their respective QTLs. Interestingly, the LOD score peaked at the position of NBS-LRR genes and the QTL explained 19.6% of the total phenotypic variation in CPR-01, as it did in CPR-02, where the QTL explained 7.9% of the phenotypic variation.

In summary, a total of 18 QTLs for ascochyta blight resistance were identified on LGs 2, 3, 4, 5, 6 and 8 explaining 6 to 33% of the total phenotypic variation in CPR-02, CPR-03 and CPR-04 under greenhouse and field conditions. Using the abundant information from previously reported genomic regions for ascochyta blight resistance, both new and common genomic regions for ascochyta blight resistance were identified. In this study, a total of 31 NBS-LRR genes co-localized with ascochyta blight QTLs in four RIL populations were identified. Eight NBS-LRR genes were found common in at least two RIL populations. The co-localization of NBS-LRR genes within QTLs was further confirmed by genetic mapping of two NBS-LRR genes in two different RIL populations.

CHAPTER 5

GENERAL DISCUSSION

CHAPTER 5: GENERAL DISCUSSION.

5.1 DISCUSSION

Identification of disease resistance genes and an understanding of the genetic interaction between plant and pathogen will aid in the development of improved resistance to ascochyta blight in chickpea. Over the past few decades, the general understanding of the plant immune system has been significantly improved as reported in several review papers (Dangl and Jones, 2001; Hammond-Kosack, K.E. and Jones, 1997; Jones and Dangl, 2006; Qi and Innes, 2013; Zhang et al., 2013). The plant immune system consists of plant disease resistance genes including NBS-LRR genes that play a major role in resistance against a diverse array of pathogens (Hammond-Kosack and Jones 1997). Conventionally, NBS-LRR genes are known to provide resistance against biotrophic pathogens through the activation of salicylic acid following host-pathogen interaction in a "gene-for-gene" or "guard" model (Glazebrook, 2005). Resistance mechanisms against necrotrophic pathogens were limited to phytotoxin production and activation of jasmonic acid (JA) and ethylene pathways (Glazebrook, 2005). Recent studies tried to establish a link between NBS-LRR genes and necrotrophic pathogen and a few of these studies reported that the activation of NBS-LRR genes leads to susceptibility against necrotrophic pathogens (Lorang et al. 2007; Nagy and Bennetzen 2008; Faris et al. 2010). Most often the response of NBS-LRR gene activation is a hypersensitive response leading to programmed cell death and since necrotrophic pathogens feed on dead cells, such pathogens take advantage of this system resulting in susceptibility. In contrast, other studies showed the involvement of NBS-LRR genes in resistance reactions against necrotrophic pathogens (Staal et al., 2008; Zhu et al., 2016). The involvement of NBS-LRR genes in providing resistance against both biotrophic and necrotrophic pathogens makes these genes an invaluable source for improving disease resistance in plants.

Using map-based cloning of R-genes, the role of NBS-LRR genes in enhancing the resistance against multiple pathogens has been demonstrated or, alternatively, their role has been proposed based on their co-segregation with genetically mapped loci for disease resistance (Marone et al., 2013). QTL analysis links complex phenotypes to genomic regions and anchoring these genomic regions to the physical map allows the identification of the candidate genes within the QTL intervals (Madrid et al., 2014). A single QTL interval may encompass several genes, therefore, the presence of homologous genes with known function in the trait of interest in model species or other crop is often used to narrow down the co-

localized genes in the QTL intervals to candidate genes. In potato, NBS-LRR sequences were found to co-localize with eleven disease resistance loci (Bakker et al., 2011). Similarly, in soybean, Kang et al. (2012) analyzed NBS-LRR genes that co-localized with disease resistance QTL and observed that about 63% of the disease resistance QTLs were located within the 2 Mb regions of each chromosome that flank the NBS-LRR genes. Thus far, no study has reported the association of NBS-LRR genes with ascochyta blight resistance in chickpea.

Genome-wide analysis identified 121 NBS-LRR genes in the 'CDC Frontier' genome. These NBS-LRR gene constitutes 0.43% of the total chickpea genome which is relatively low compared to *Glycine max* (0.58%), *Medicago truncatula* (0.66%), and *Arabidopsis thaliana* (0.75%; Ameline-Torregrosa et al. 2008; Meyers et al. 2003; Kang et al. 2012). Despite a low number of NBS-LRR genes, most chickpea NBS-LRR genes function as independent R-proteins as they encode essential conserved domains. Among the 121 NBS-LRR genes, 98 NBS-LRR genes encode proteins with both NBS and LRR domains and the remaining 23 genes were incomplete as they lack the LRR domain. Similar to other dicot species, chickpea contained both TNL class and CNL class genes and phylogenetic analysis results indicated the diversified evolution of the TNL class from non-TNL/CNL classes. Chickpea NBS-LRR genes were classified into eight classes based on the domain architecture. The chickpea NBS domain consisted of eight major motifs in strict order, six conserved motifs (P-loop, Kinase-2, RNBS-B, RNBS-C, GLPL and MHDV) were consistent in both CNL and TNL classes and two diverse motifs (RNBS-A and RNBS-D) distinguished the CNL and TNL classes. The NBS-LRR genes were randomly distributed across all chickpea chromosomes and nearly half of the NBS-LRR genes (48%) were present in mono-clusters and mixed clusters which reflect that these genes might have evolved through tandem duplications. In this study, the previously reported QTLs for ascochyta blight were anchored to the physical map of 'CDC Frontier' based on the physical position of the flanking STMS markers. Thirty NBS-LRR genes were co-localized with the physical position of the nine ascochyta blight resistance QTLs on chromosome 2, 3, 4, 6, and 8. Expression analysis using real-time qPCR showed differential expression in at least one genotype at one-time point compared to mock-inoculated control sample in 27 NBS-LRR genes co-localized with the ascochyta blight resistance QTLs. Few NBS-LRR genes showed genotype-specific expression pattern.

This study builds the foundation for future research since a significant number of NBS-LRR genes were present within the previously reported QTLs for ascochyta blight resistance and

differential expression of these co-localized genes indicated the potential of NBS-LRR genes as candidate genes for ascochyta blight resistance. This study also provides the genomic resources for further functional studies to validate the association of NBS-LRR genes with other diseases in chickpea.

Another study was conducted to identify common and potentially new genomic regions associated with ascochyta blight resistance in three RIL populations derived from a cross between a common susceptible parent 'ICCV 96029' and parents with moderate resistance to ascochyta blight ('Amit', 'CDC Luna', and 'CDC Corinne'). The study also identified co-localized NBS-LRR genes with the QTLs for ascochyta blight resistance in the RIL populations, and genetically mapped the selected NBS-LRR genes to validate their co-localization.

QTL mapping was conducted in three RIL populations using SNP-based genetic maps under greenhouse and field conditions. Eighteen QTLs for ascochyta blight resistance were identified on LGs 2, 3, 4, 5, 6 and 8, each accounting for a range of 6-33% of the total phenotypic variation. The environment plays a significant role in disease development, particularly in the case of ascochyta blight, and environment specific QTLs were observed in previous studies (Sabbavarapu et al., 2013). Similarly, in this study environment-specific QTLs were identified due to the variable environment in different years and under greenhouse and field conditions. However, some common QTLs were also observed in the field in different years, and under greenhouse and field conditions. Comparison by anchoring QTLs to the physical map indicated most of the QTLs were physically mapped on common genomic regions on chromosome 2, 3, 4, 5, 6 and 8 which suggests that despite the use of different resistance sources, screening methods and different environments there are some common genomic regions that are involved in providing resistance to ascochyta blight in chickpea. Furthermore, a few new QTLs were also identified on chromosome 3, 5 and 8. In this study, 32 NBS-LRR genes were co-localized within the physical position of 16 QTLs associated with ascochyta blight resistance in three RIL populations from our study and in a RIL population studied by Daba et al. (2016). These co-localized genes belong to different classes of NBS-LRR genes and both single gene and clusters of NBS-LRR genes were found within the QTLs for ascochyta blight resistance. The reason behind identifying the co-localized NBS-LRR genes in these RIL populations was mainly to confirm the physical position of NBS-LRR genes in ascochyta blight resistance QTL interval through genetic mapping. Previously reported QTLs were used to identify the co-localized NBS-LRR genes

based on their physical location. The genetic map of two NBS-LRR genes in QTL (*qAB-1-4.1*) in the CPR-01 population and one NBS-LRR gene in QTL (*qAB-2-2.1*) in CPR-02 confirmed the physical co-localization of these NBS-LRR genes in their respective QTLs.

The presence of NBS-LRR genes in the genomic region involved in ascochyta blight resistance provides an initial support for the involvement of chickpea NBS-LRR genes in ascochyta blight resistance. However, other genes present within the QTL interval and outside the confidence interval may also be involved in disease resistance mechanisms (Madrid et al., 2014). For example, a cluster of three CNL class genes and two candidate genes *CaETR1* and RLK genes were found to co-localize with several QTLs on chromosome 4 and these genes are involved in plant defense responses. *Ein3* is a plant-specific transcription factor which mediates ethylene responses (Madrid et al., 2014). The RLK genes are homologs of *A. thaliana* PBL13, a serine/threonine RLK class gene which enhances a reactive oxygen species burst to provide disease resistance (Li et al., 2017). Plant NBS-LRR proteins act through a network of signalling pathways and trigger a series of plant defense responses, such as the activation of an oxidative burst, the induction of pathogenesis-related genes and a hypersensitive response (McHale et al., 2006). Several signaling molecules in the plant defense responses including ethylene, salicylic acid, and jasmonic acid are involved downstream of NBS-LRR proteins and there is complicated cross talk between the different signaling pathways involving both synergism and mutual antagonism between pathways (McHale et al., 2006). In *A. thaliana*, at least three independent signalling pathways are induced by NBS-LRR proteins and each class of NBS-LRR genes tends to signal through different downstream pathways (McHale et al., 2006).

QTL mapping identified genomic regions containing one or more candidate genes and the DNA polymorphism between the alternate alleles of one of the candidate genes that could change the protein product can elucidate the involvement of the candidate genes in that trait of interest (Mackay et al., 2009). On chromosome 2, most of the ascochyta blight resistance QTLs identified in the previous studies and from the current study are clustered in the same genomic region where three NBS-LRR genes and the previously reported candidate gene (*Ein3*) are co-localized. No polymorphism was identified within the coding region of *Ein3* between the resistance (ILC3279) and the susceptible parents (WR315), limiting the mapping effort in the intraspecific cross and usefulness in MAS (Madrid et al., 2014). However, the candidate gene *LOC101493700* from the CNL class was polymorphic between the resistant parent ‘Amit’ and the susceptible parent ‘ICCV 96029’, and a SNP polymorphism was

located in the gene coding region. Therefore, in contrast to *Ein3*, this gene was successfully mapped in the CPR-02 population and the QTL (*qAB-2-2.1*) peak was located to the *LOC101493700* locus. This gene-based marker can be directly used for MAS and for screening germplasm for ascochyta blight resistance.

Functional studies are essential to test the involvement of candidate genes in the trait of interest as the limitation of QTL mapping is that the precise location of the genomic region involved in the trait of interest is difficult to determine, despite the large size of populations and numerous genetic markers. For example, although the candidate gene *CaETR1* reported by Madrid et al. (2014) was co-localized in $QTL_{AR1,}$ this gene was not differentially expressed in the transcription profiling study conducted by Coram and Pang (2006). Therefore, in this study the expression profiling of NBS-LRR genes co-localized with ascochyta blight resistance QTLs was conducted. The results showed that 27 NBS-LRR genes co-localized with nine ascochyta blight resistance QTLs showed differential expression in response to *A. rabiei* inoculation in both resistant and susceptible genotypes when compared to the mock-inoculated samples. This indicated the potential involvement of these genes in response to ascochyta blight infection. Early up-regulation of the NBS-LRR genes was observed in the susceptible genotype and few genes showed genotype-specific expression patterns. These results provide an indication for the functional involvement of NBS-LRR genes upon ascochyta blight infection. However, further functional and expression studies are required for characterization of NBS-LRR genes function in chickpea using over-expression or silencing of these genes to fully understand their role in providing resistance to ascochyta blight.

Further research efforts are required to narrow down the list of candidate NBS-LRR genes involved in ascochyta blight resistance. Functional characterization of NBS-LRR genes which showed genotype specific expression, particularly the two NL class genes *LOC101509145* and *LOC101498915* which only showed up-regulation in moderately resistant cultivar "CDC Corinne" will help to understand their role in providing resistance to *A. rabiei*. The NBS-LRR gene clusters were found co-localized in major QTLs on chromosome 5, such as in CPR-02, a cluster consisting of two TNL class genes *LOC101497758* and *LOC101498509* was found co-localized within the major QTL qAB-*2-5*.2 on LG5 which explained 33% of phenotypic variation. Independently, the same TNL genes were also found co-localized within QTL qAB-*4-5.1* in CPR-04 on LG5 which explained 16.5% of phenotypic variation. Therefore, it will be interesting to further analyse

such genes as the largest gene clusters of NBS-LRRs were located on chromosome 5 and these genes were linked to genomic regions invovled in provinding resistance to ascochyta blight. Further in depth studies are required to chracterize the NBS-LRR genes which were genetically mapped in CPR-01 and CPR-02 as in both populations the QTLs peaked at the position of NBS-LRR gene based markers. It will be interesting to study how these polymorphism in NBS-LRR genes effect their biological function in susceptible and moderately resistant chickpea cultivars to ascochyta blight.

These results provide the first in-depth molecular characterization of NBS-LRR genes in the chickpea genome providing potential candidate genes for ascochyta blight resistance. More importantly, this work has built the foundation for future studies to intensively study the role of NBS-LRR genes in ascochyta blight and several other biotic stresses in chickpea.

5.2 CONCLUSION

Results from the current study provide circumstantial evidence that NBS-LRR genes are involved in the resistance mechanism against ascochyta blight infection in chickpea and these genes are potential candidate genes for ascochyta blight resistance. The rationale for drawing this conclusion is based on the results that NBS-LRR genes were found to co-localize within the physical position of the previously reported QTLs and in QTL regions identified in four RIL populations in the current study. Genetic mapping of the NBS-LRR genes in two different RIL populations validated their co-localization within the ascochyta blight resistance QTLs. Most importantly, differential expression of the co-localized NBS-LRR genes with ascochyta blight QTLs in three chickpea cultivars with different levels of resistance upon infection with *A. rabiei* when compared with control samples, suggests that NBS-LRR genes are functional candidate genes for ascochyta blight resistance. However, further studies on over-expression or silencing of these genes are required to confirm their function in resistance to ascochyta blight. This study provided an insight into chickpea disease resistance gene architecture and built the foundation for future studies by providing a genomic resource to study the association of NBS-LRR genes with resistance to different diseases of chickpea. The markers closely linked to the ascochyta blight QTLs can be used for MAS and pyramiding different QTLs for breeding improved resistance to ascochyta blight in chickpea.

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APPENDIX 1: PRIMER SEQUENCES OF THE CO-LOCALIZED NBS-LRR GENES AND REFERENCE GENES USED FOR REAL-TIME PCR.

GENE ID	Class	PRIMER SEQUENCE
LOC101493700	*TNL*	F 5'-TAATCAGCATTCTTCTTCTCCATCT-3'
		R 5'-AACGCCAAATCAAATTCCCTATC-3'
LOC105851141	*TNL*	F 5'-GGATGTGCGTCATAAGTCAGA-3'
		R 5'-GTAGGAGAATCCATCCCAACTAAA-3'
LOC101498642	*TNL*	F 5'-GTCCCAACCAACTTGTTGAATTA-3'
		R 5'-GGGCAAATACTTCTTGTCTTTCC-3'
LOC101488503	*TNL*	F 5'-TCAAGAAATGGGCAGGGATATT-3'
		R 5'-TGCTTCAGTTCCCTTGTTTCT-3'
LOC101494533	*TNL*	F 5'-AGGCTCAGAGGATGGAGAA-3'
		R 5'-CCTGGAGCAATCTGTGAGTATTA-3'
LOC101497058	*CNL*	F 5'-GAACTGGAGACCAATCAGAAGG-3'
		R 5'-GGAGAAACAGGGACAAGATGAG-3'
LOC101496750	*CNL*	F 5'-TACGCTCGACGCAATCAATAA-3'
		R 5'-TCCCAAATTCAGCAGGGAATAG-3'
LOC101497042	*CNL*	F 5'-CAACTCACCACCACTCTTCTT-3'
		R 5'-TGGCCTCTATGTCTTGATTTCTAC-3'
LOC101504229	*CNL*	F 5'-CTTCCACCACTTGGACAATTAC-3'
		R 5'-GGGAGGGAAAGATAATTCCTGA-3'
LOC101509145	*NL*	F 5'-TTGAAATTGATAGACCTCCCAAAC-3'
		R 5'-CAGCAGCAACATCTAATTCTTGA-3'
LOC101501248	*NBS*	F 5'-CGGCGTGATAGGTCAGTTAAG-3'
		R 5'-GCTCTTGTTGCAGCCAAATTA-3'
LOC101502198	*NBS*	F 5'-CAGCTGCAAGGTGAGAAGTAT-3'
		R 5'-GGTTGAATGACCCTGAGTTGTA-3'
LOC101505313	*TNL*	F 5'-GGCTTTGTTGGCATGGATTT-3'
		R 5'-CCCTTCAGAAACTGAGACTTCC-3'
LOC101498365	*NL*	F 5'-GGAAGAGAGAGTGACAGGAAAG-3'
		R 5'-AGCCACCAATTCCCACTATG-3'
LOC101498707	*CN*	F 5'-CCGAGCAAGCAAGACATTTG-3'
		R 5'-ACCCACTCTATCAATGGGAAAC-3'
LOC101499030	*CNL*	F 5'-TAGCGGTAGACTCGCATACT-3'
		R 5'-CAACTCCGAGGACAGGAAATAG-3'

LOC101495647	*CNL*	F 5'-GAAAGCACAATGGCAAGGTC-3'
		R 5'-CAGGGTCGGGTTTGAGAAATA-3'
LOC101492873	*NBS*	F 5'-CCTAAGACAGAGAATGCCACAA-3'
		R 5'-CAACTGTGGTGACTGTGAAGA-3'
LOC101500245	*CNL*	F 5'-GCTTCTGAGGAACAACTGGTTA-3'
		R 5'-ACGGCAAGGTCGTGAATAAG-3'
LOC101502375	*TNL*	F 5'-TGATATGCACAAGGTGGATGTAG-3'
		R 5'-CACCTGAGAAGACGGCATAAA-3'
LOC101505907	*CN*	F 5'-GTTGGCTTTGTCCATGAATCTG-3'
		R 5'-CCCAATCCACCCATACCAATAA-3'
LOC101492735	*CNL*	F 5'-TGGTCTCCCTCTAGCTGCAA-3'
		R 5'-ACCTTCCCACTCTCTAACATCT-3'
LOC101511908	*TNL*	F 5'-GGGCAGTGCCTCTGATGTAT-3'
		R 5'-GCTTAGCTCTGCAACTGGCT-3'
LOC105851158	*TNL*	F 5'-TAGTAGGTTGTGGCGTCCTG-3'
		R 5'-CTTTACAACTTCTGTTCCCTTATGC-3'
LOC101495691	*TNL*	F 5'-GGATTGCTGGATTGGTGGCT-3'
		R 5'-CCAACTTGACTTTCCCAGGC-3'
LOC101505949	*RN*	F 5'-TGTCCATGAGATAGTGAGAGGT-3'
		R 5'-GCCACTTCTCAAAAGGCTGC-3'
ACTIN	*Act1*	F 5'-CCTGATGGACAGGTGATCAC-3'
		R 5'-GGAACAGGACCTCTGGACATCT-3'
ELONGATION FACTOR	*Ef1α*	F 5'-TCCACCACTTGGTCGTTTTG-3'
		R 5'-CTTAATGACACCGACAGCAACAG-3'
GAPDH	*GAPDH*	F 5'-CCAAGGTCAAGATCGGAATCA-3'
		R 5'-CAAAGCCACTCTAGCAACCAAA-3'
INITITAION FACTOR	*IF4a*	F 5'-TGGACCAGAACACTAGGGACATT-3'
		R 5'-AAACACGGGAAGACCCAGAA-3'
RIBOSOMAL RNA	*18SrRNA*	F 5'-ACGTCCCTGCCCTTTGTACAC-3'
		R 5'-CACTTCACCGGACCATTCAAT-3'

APPENDIX 2: HISTOLOGICAL STUDY TO VISUALIZE *ASCOCHYTA RABIEI* STRUCTURES AT DIFFERENT TIME POINTS IN GENOTYPES WITH DIFFERENT LEVELS OF RESISTANCE

A.2.1. PLANT MATERIAL

The samples collected from three chickpea genotypes: 'CDC Corinne', 'CDC Luna' (both moderately resistant to ascochyta blight) and 'ICCV 96029' (susceptible) after *A. rabiei* isolate AR-170 inoculation at four-time points at 12, 24, 48, and 72 hours post inoculation (hpi) for the expression analysis in the first study were also used for fungal light microscopy in the greenhouse trial.

A.2.2. METHOD

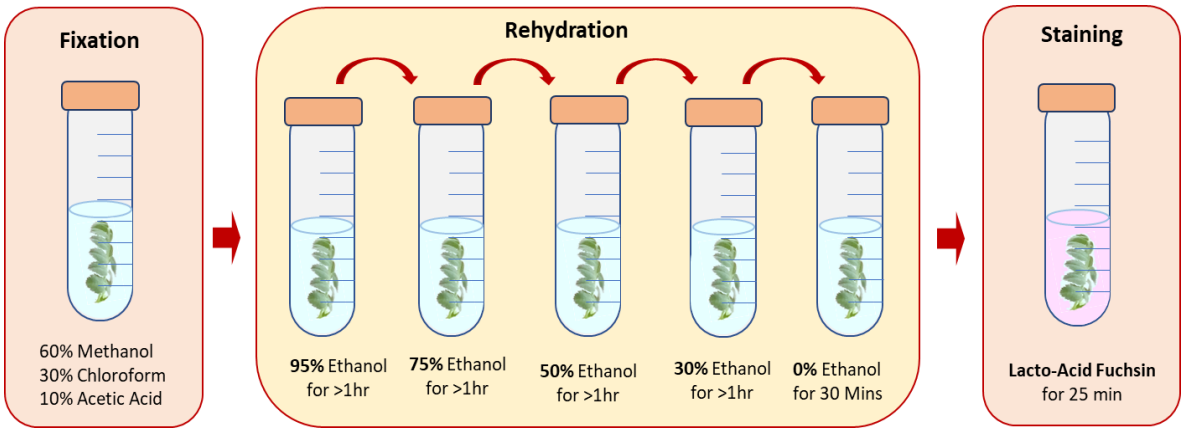

Figure A.1.1. The lacto-acid fuchsin staining method used for light microscopy to clear plant tissues and stain fungal structures for visualization.

A.2.3. RESULTS

Different stages of *A. rabiei* were observed under a light microscope after lacto-acid fuchsin staining of three chickpea genotypes: 'CDC Corinne', 'CDC Luna' and 'ICCV 96029' at four-time points at 12, 24, 48, and 72 hours post inoculation (hpi, **Figure A.1.2**). In each genotype, *A. rabiei* spores started germinating at 12 hpi and more than 50% spores were germinated and started elongation phase by 24 hpi. Penetration through stomata was observed but appressorium formation and penetration through appressoria was not evident. Pathogen growth was observed at later time points but genotypic differences were not observed.

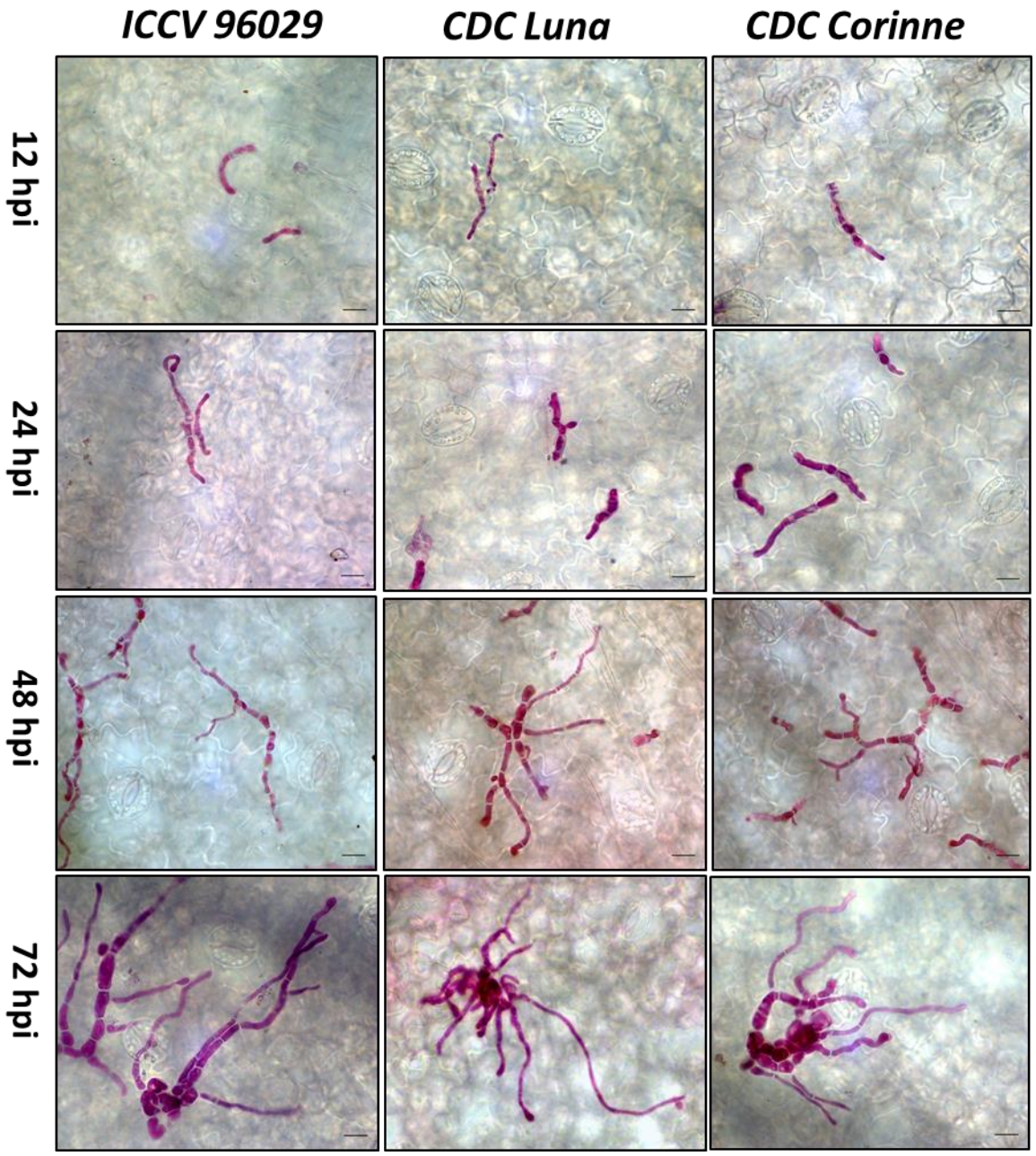

Figure A.1.1. Different stages of *Ascochyta rabiei* observed under a light microscope from three chickpea genotypes: 'CDC Corinne', 'CDC Luna' (both moderately resistant to ascochyta blight) and 'ICCV 96029' (susceptible) at four-time points at 12, 24, 48, and 72 hours post inoculation (hpi).